

10 January 1980

# Biotechnology back in the limelight

A FEW years ago — in the mid to late 60s — there grew up a fashion for thinking that the 'new biology' would revolutionise the chemical and pharmaceutical companies. It has made its impact, but not so great as was first promised. Now again the genie rises, to face a number of problems — which we outline on pages 122-131.

The first flowering came with the elucidation of the sequences, structure, and function of a few enzymes, and their immobilisation to act as efficient, recoverable industrial catalysts, so that it became conceivable that all biology could be tamed and put to work. It affected mainly enzymologists and microbiologists. The second follows the discovery by molecular geneticists of techniques for manipulating and transferring DNA between organisms, and it has led to that new group of biologists discovering something quite different: the profit motive and the ways of industry.

Problems and acrimony have arisen between the biologists and industry, and among the biologists, particularly over the question of confidentiality. Scientists with one foot in the academic and one in the business world have felt a conflict between the need to publish fast and first in the former, and to keep secrets and respect patent law in the latter. In some of the smaller business ventures the urge to publish appears to have been effectively quashed; in others, it survives, if not completely intact.

Secrecy means that some academics cannot disclose to their colleagues the details of industry-funded research that is going on in the lab next door — souring relationships between them. For example the silence from most of the laboratories competing fiercely to sequence, clone, and manufacture interferon has, at times, been complete. But that is not surprising when what used to be an innocent amino-terminal acid sequence can be the key to a fortune.

These problems, though, should be temporary. They come about because the firms need to draw on a new community of scientists, one that is used only to the priorities of basic research. But already a new breed of molecular biologist is emerging which concerns itself not so much with understanding basic mechanisms as with manipulating them to reach desired goals. The joys of mission-oriented research are totally different from those

of simple inquiry, and slowly they will sort out a different kind of biologist, and eventually a different career structure, different opportunities and relationships. It was in just this way that the devices engineer became separated from the solid state physicist, and the nuclear power specialist from the physicists who play with quarks. The same will happen in biology.

The middle-term problem rests with governments: how to set up a productive biotechnological community involving both basic biologists and industry, so that interested biologists can 'get their feet wet' and solutions be found to real practical problems. As with any technology, there will always be a need for an academic bioengineering community dealing with long-range issues of only partial interest to industry, between the industry-based biologist and his or her fundamentally inclined colleague; it is for government — and the universities — to set up this community and ensure its proper recruitment, training, and regeneration. In the UK, it is to be hoped that the Royal Society committee currently inquiring into biotechnology will make recommendations on this matter.

But strangely the long-term issue rests again with the biologists. The physicists have learned, rather late, that their technologies have had an immense impact on society. That impact began with a certain selection of priorities, mainly by industry, which led physicists to research in one area rather than another. (Thus, for example, solid state physics was vigorously pursued because of its significance to the electronics industry, whereas oceanography was relatively neglected — until the need to detect nuclear submarines or search for minerals.) The same forces are undoubtedly at work now in biotechnology — and this is precisely the moment when patterns of research will be formed which will ultimately shape lives. Communities and laboratories will be set up in various targeted disciplines, some fields will be encouraged and others neglected; expertise and training will develop along lines which will later be difficult to change. It is an interesting moment for biologists: they have great power in their hands. Do they let the entrepreneur guide them, willy nilly, to the fastest return? Or do they, if ever so slightly, change his priorities? □



## United States

David Dickson reports from the American Association for the Advancement of Science's annual conference in San Francisco.

# New effort urged to control nuclear weapons spread

RECENT events in Iran and Afghanistan, and the domestic political response to these events in the US, have increased the need for scientists to support efforts to control the proliferation of nuclear weapons, a succession of speakers told a forum on ending the nuclear arms race.

Talking two days after President Carter had requested the US Senate to postpone its debate on the ratification of the Strategic Arms Limitation Treaty (SALT II), the speakers warned of the dangers of linking the nuclear weapons issue to broader political considerations of East-West relations.

"The present crises show that no security has been gained by massive nuclear arsenals. In fact the situation is only made more dangerous," said Professor Everett Mendelsohn of Harvard University. "I believe that scientists, who have been so especially involved in the design and construction of nuclear weapons and advanced missile systems, have an added responsibility to press for nuclear disarmament now. The scientific community has been too quiet for too long on the nuclear arms race."

Jeremy Stone, director of the Federation of American Scientists, emphasised that the importance of separating SALT from detente, as well as the whole logic of arms control, had never been so clear. "Supporters of SALT will have to, and

should, separate their case for SALT from their case for detente. And SALT opponents will have to consider how far they want to go in giving up hopes for reducing the risks of nuclear war in order to ensure sharp US responses to Soviet adventures."

Reflecting the shifting political climate with respect to arms control, and military spending Dr Bernard Feld, editor-in-chief of the *Bulletin of the Atomic Scientists*, pointed out that the magazine had recently decided to move the clock on its front cover, an indicator of the world's proximity to nuclear destruction, from nine to seven minutes before midnight.

"This seemed an adequate gesture a few months ago, but after the recent events we should probably move it even closer, perhaps to five minutes to midnight," said Dr Feld. Reasons for the decision to shift the clock, which had previously remained unchanged since 1974, included the apparent breakdown in the SALT process and the spread of new nuclear weapon systems in Europe.

Professor Mendelsohn told the audience that a new *ad hoc* committee was being established to coordinate the lobbying efforts of scientists in pushing for nuclear weapons control, and urged that the AAAS council adopt a resolution recognising the importance of continued support for the SALT treaty. □

## 'Big brother' approach to space must go

THE US should support the efforts of the United Nations to forge an international consensus on the commercial uses of space, as this was the only way to guarantee long-term access to the markets being opened up by current research, according to Dr Jerry Grey, administrator of public policy for the American Institute of Aeronautics and Astronautics.

Referring in particular to a draft treaty of the uses of the moon approved by the UN General Assembly in New York last month, Dr Grey said that it was important for the US Senate to ratify this treaty, even though many scientists and industrialists had already expressed concern about some of the restrictions that it might place on the space activities of US corporations.

"If necessary, the US should express reservations in the process of ratifying the treaty. But we must hedge our bets as effectively as possible to ensure that our long-term interests are protected. We cannot afford to have the US barred from these activities as a result of charges of imperialism or of exploitation of the Third

World," said Dr Grey.

He pointed out in particular that it was the US which had introduced the 'common heritage' language into discussion about the uses of space when the UN had first started discussing the issue in the early 1970s. Any other position than accepting the common heritage principle "would have seriously prejudiced US ability to conclude the cooperative agreements which are essential to US participation in potentially lucrative global markets," Dr Grey said. And awareness of this need among negotiators was one welcome sign that the US might be revamping its present "big brother" approach to global space applications.

In addition to the UN discussions, the recent successes of European space efforts emphasised how formerly sacrosanct US markets were being invaded. Unless policy-makers reversed their traditional reluctance to engage in cooperative activities, the US would almost certainly lose out on major forthcoming market opportunities. □

## EPA wants more data on chemicals

UNLESS the chemical industry is prepared to present more substantial data on the health and environmental effects of new chemicals, the Environmental Protection Agency may have to take various measures — including if necessary temporarily preventing the manufacture of the chemicals in question — to ensure that adequate testing has been carried out, according to Dr Steven Jellinek, director of the EPA's office of toxic substances.

Dr Jellinek said that since the pre-manufacture notification requirement of the Toxic Substances Control Act had come into force six months ago, data on the testing of 51 new chemicals had been passed to EPA for approval. In examining these notices the agency had in particular found a tendency for companies to go for less expensive forms of tests, resulting in a relative lack of what EPA considered to be adequate safety information.

"In many cases, very little environmental or health data is included — for example none of the notifications received included data on chronic testing," said Dr Jellinek. "Unless the situation is corrected, our review of such notices is going to be based on inadequate data, and we will have to rely on our own studies, such as the use of short-term screening tests, which will mean that the information on which we base our decisions is very uncertain."

Dr Jellinek emphasised that it was not the intent of the EPA to stifle technological innovation through excessive regulation — indeed in many cases regulation had had the effect of stimulating money-saving improvements to production processes. However, in considering the objections of the chemical industry to the agency's activities, it had to be recognised that major breakthroughs in the industry were rare and that most innovation was of a relatively run-of-the-mill variety, which did not necessarily require the same reverential treatment.

However Dr Jellinek said that the agency was considering various ways in which it could "fine-tune" its regulatory efforts, including the possible exemption of certain little used chemical substances from the full requirements of legislative controls, and enabling generic pre-manufacturing notices to be granted on a range of closely-related products.

Earlier in the session, Dr William J McCarville, director of environmental affairs for the Monsanto Company, demanded the separation of scientific decisions from political decisions in the regulatory process — and suggested the creation of a high-level independent scientific panel to answer the scientific needs of all federal agencies that regulate chemical carcinogens. □



## United Kingdom

## Ex-energy secretary criticises decision-making

FORMER UK Secretary of State for Energy, Mr Tony Benn, has broken the unwritten rules on public exposure of parliamentary procedures by giving an extended interview to Granada television (*World in Action* shown on 7 January) about key decisions taken during his 11 years as a senior Cabinet Minister. According to *World in Action* reporters, Whitehall officials have refused to respond to his criticisms on the grounds that he is breaking a long standing tradition that disputes should be kept behind closed doors.

Mr Benn cites five important issues on which, as the person in charge of Britain's nuclear programme, he was either not informed, misinformed or actively opposed by government bureaucrats.

**The 1978 AGR-PWR decision:** Benn favoured the British designed advanced gas cooled reactor (AGR) over the US designed pressurised water reactor (PWR) — of the type that failed at Harrisburg — on the grounds of safety. He told *World in Action* that he asked his civil servants to prepare a Cabinet paper in favour of the AGR. Instead, in conjunction with industrialists like Sir Arnold Weinstock of the General Electric Company who had

obtained the UK license to build PWRs, they argued that the PWR had better export potential to countries like Iran. His department, he claims, then refused to help his political advisors prepare a brief in line with his views, and the Central Policy Review Staff (think tank) circulated a paper to other ministers directly opposing them. In this case Benn won the argument in favour of AGRs because he "was also making great efforts to see that other ministers knew what this battle was about."

**The major Windscale leaks:** In 1976, during discussions of expansion of the Windscale nuclear reprocessing facility, Benn heard about the major silo leak from the newspapers. In March last year, he responded to the 30,000 curie-300rem/hr sump leak by calling for a public inquiry in spite of what he describes as "intense political pressure" by the bureaucrats.

**The UK-Namibian uranium deal:** In 1970, officials in the Department of Energy approved contracts to let the UK nuclear industry obtain uranium from the Rossing mine in Namibia. Namibia was illegally occupied by South Africa and the Labour Government had insisted that it be

informed before any contract was signed. The cabinet was not informed of the department's action by what Benn describes as a frequent bureaucratic ploy. Signed contracts are placed in "one or two or perhaps three red boxes a night" without drawing the ministers attention to their importance.

**The 1968 theft of 200 tonnes of uranium from Euratom:** The theft, from the ship Sheerbourg, bound for Genoa from Antwerp by presumably Israeli agents, was not reported to Benn when he was Energy Secretary on the grounds that Britain was not a member of Euratom.

**The Russian Kyshtym nuclear disaster in the 1950s:** This was not made public until 1977, although Benn claims that the information was known to the CIA who passed it on to the UK Atomic Energy Authority. The UKAEA did not tell Benn about the accident because, he claims, it feared that the accident "might throw doubt upon the safety of nuclear operations worldwide."

Benn feels that the only remedy to "the state within the state" is to expose the operation of government to public debate.

Joe Schwartz

## NEWS IN BRIEF

## Japan produces enriched uranium

THE Japanese government announced on 26 December that it had produced enriched uranium from its own factories. Previously, Japan has relied on the US for its supplies of enriched nuclear fuel. A factory in Ningyotoge in the western part of the country has produced 300 kg of uranium with a 3.2% concentration of  $^{235}\text{U}$  the fissionable isotope of uranium. ( $^{235}\text{U}$  has a natural abundance of 0.72%). By 1981 the country is expected to produce 50 tonnes of enriched fuel annually. The uranium will be used to fuel Japan's rapidly expanding nuclear programme which envisages the construction of an additional 15 power plants in the next five years, a doubling of present capacity. Enriched uranium at this level can also be used to construct fission bombs.

## Evidence on laboratory animals invited

A UK House of Lords select committee which will consider revision of the Cruelty to Animals Act of 1876 has invited written evidence from interested individuals and organisations on current practices in the use of laboratory animals. The committee seeks answers to three questions:

- Is present law and administration for controlling the number and use of laboratory animals unsatisfactory?
- If present law needs amendment, what changes ought to be made?
- Does the proposed House of Lords Laboratory Animals Protection Bill meet the need for reform?

Evidence should be sent before 24 January 1980 to the Clerk, Select Committee on the Laboratory Animals Protection Bill, House of Lords, London SW1 0PW.

## Neurochemistry society wins action

THE International Society of Neurochemistry has won its legal action against Pergamon Press to prevent it from continuing to publish a journal with the same name as the society's journal. The dispute arose when the society moved the *Journal of Neurochemistry* from Pergamon to Raven Press earlier this year. After completing a ten year contract with Pergamon, the society sought another publisher because of dissatisfaction with Pergamon's "long publication times" and the "inadequacy of its accounting procedures". Pergamon had insisted that it had the right to publish a journal of the same name and had sent a mailing to sub-

scribers calling for continued subscription to the journal. It had also held back 50 manuscripts whose copyright is held by the society and had refused to release the subscription list. Pergamon now will hand over the subscription list and the unpublished manuscripts and will refrain from publishing any journal under the title "Journal of Neurochemistry". The society now will seek recompense for the 800 non-renewed subscriptions due to the dispute and for its costs in communicating with subscribers about the dispute.

## Former Volvo director appointed head of ESA

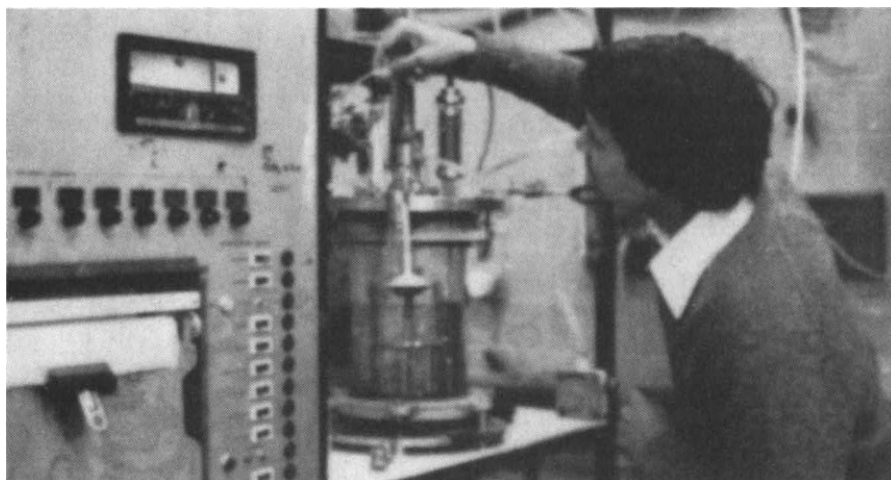
THE Council of the European Space Agency appointed Mr Erik Quistgaard to the post of Director General at its 19 December meeting. Mr Quistgaard, aged 58 and a Danish national, graduated as a mechanical engineer (MSc) from the Technical University, Copenhagen. He spent three years with the Chrysler Corporation in the US and was General Manager and later director of Volvo in Sweden. After leaving Volvo he became Managing director of Odense-Lindo Stalskibsvaerft A/S, the Danish shipbuilding firm. Quistgaard will replace ESA's present director, Mr Roy Gibson, on 15 May 1980.



# The biology business

In the first two articles of our special section, we look at the industrial potential of biotechnology and who is likely to exploit it first.

**Professor Brian Hartley** of Imperial College, London is optimistic that it will be applied rapidly to many industries — he outlines its promise below. And opposite, **Robert Walgate** compares the efforts of different countries throughout the world



*Tuning fermentation conditions before pilot plant trials*

## The bandwagon begins to roll

WHY should industry and governments think seriously of investing in biotechnology?

First is the tremendous rate of development of the technology itself. Five years ago, even optimists would have predicted that it would be a decade before genetic engineering could be commercially exploited, but this is about to be disproved. The earliest prospect is for new pharmaceuticals made cheaply by fermentation. Human hormones, enzymes or antibodies will replace animal products or create new therapies. Already bacterial fermentation processes for human somatostatin, growth hormone and insulin are patented and there is a world-wide chase for cheap interferon, potentially the wonder-drug of the 80s. Less dramatic, but equally important, is the prospect of new, cheap, safe and effective vaccines. By separating the gene for a viral antigen from the toxic or infective components, this harmless protein could be made cheaply by a microorganism. Such inherently safe products should also reduce expensive toxicity testing. Already progress is reported towards hepatitis B and influenza vaccines, and one can envisage that even measles and the common cold may succumb. Cheap animal vaccines will increase agricultural productivity: the prospects of such vaccines for foot and mouth disease or swine fever now merit consideration of programmes to eradicate these diseases.

Genetic engineering also offers new diagnostic tools to medicine. Enzyme or antibody diagnostic kits for the general practitioner are already a thriving business, and genetic engineering promises novel and cheaper products. Tiny amounts of the DNA or RNA of viruses and

microorganisms can now be readily mapped, isolated, sequenced and replicated. Thus new subtypes of viruses can be recognised by their different DNA sequences and "probes" of DNA complementary to these will be invaluable in diagnosis and epidemiology.

The second major factor favouring biotechnology is the dramatic shift in terms of trade, and in public opinion, from fossil fuels to renewable energy resources. Environmental pressures have also forced up the costs of disposal of agricultural and industrial wastes: biotechnology can help by converting these into useful products. The simplest conversion is into microorganisms for animal feed (Single Cell Protein: SCP). For example, a British Petroleum process utilises n-alkanes — a side product of oil refining; a Shell process uses methane, also frequently wasted in oil recovery, and Imperial Chemical Industries grow organisms on methanol obtained from methane. Only the latter is currently judged economic, and then only by virtue of large-scale centralised production.

As petrochemicals become more expensive there are even brighter prospects for biotechnology in production of bulk chemicals. Japan has led the way with a profitable fermentation industry producing amino acids, nucleotides and organic acids, but this relies largely on conventional microbiology, genetics and fermentation feedstocks. It can certainly be challenged by new biotechnology applied to new sources of biomass. Indeed since many microbial products — proteins, polysaccharides, organic acids, for example — could be produced more cheaply than petrochemicals, should we not visualise new biopolymers,

biosolvents, and biodetergents?

In this light, enzyme technology will surely take off. Genetic engineers already have ways to persuade microbes to superproduce enzymes of choice and even to secrete them into the medium. Alternatively the superproduced enzymes can be retained within the cell, so that the immobilised cell itself becomes a fixed catalyst. An early example is the ICI process for producing high fructose syrups with immobilised glucose isomerase. This has received the EEC seal of approval by earning a tariff because it competes effectively with sugar beet. Enzymes embedded in membranes can form enzyme electrodes for continuous monitoring of flow processes, and one can foresee membranes that are specifically permeable to a single compound.

Not least is the contribution that biotechnology may make to the world's energy problem. In hot, sticky countries such as Brazil, big schemes are already afoot for producing ethanol from sugar cane or cassava, as a fuel for cars. Moreover big improvements on the current conventional fermentation can be expected; for example, a rapid continuous fermentation by organisms living at high temperatures that would harness the heat of fermentation to distil the ethanol. Thus as the price of oil continues to soar, the Brazilian adventure seems to be far from a risk. Indeed, Western developed countries are beginning to examine their own resources of biomass for energy; industrial and agricultural wastes are an obvious target and, just around the corner are processes for converting cellulose into liquid or gaseous fuels. Then a really balanced alternative to our oil-based economy will have arrived. □

# Who's ahead, who's behind?

Between 1967 and 1971, when immobilised enzymes were emerging as the first wave of the new biobusiness, 30% of world biotechnological patents originated in one country: the UK. But of the patents delivered since 1977, 124 originated in Japan, 39 in the US, 9 in the USSR, 8 in West Germany, 7 in France, one in Denmark, and just one in the UK.

Armed with this rather depressing information for Europe, the European Commission requested two biologists — Dr Artur Rörsch of Leiden State University, The Netherlands, and Dr Daniel Thomas of the University of Compiègne, France, to produce reports\* on the status and potential of genetic manipulations in applied biology, and of the production and exploitation of biological catalysts.

Meanwhile in France, Professor Francois Gros, Director of the Institut Pasteur, Professor François Jacob, Nobel laureate and also at the Institut Pasteur, and Professor Pierre Royer of the Delegation Generale pour la Recherche Scientifique et Technique were producing a report\* on biotechnology for the President of the Republic, Giscard d'Estaing.

All these reports carry reviews of the state of the art in biotechnology around the world; together they provide a world-wide picture.

## Japanese domination

Japan has taken an incontestable lead in biotechnology. The company Tanabe Seiyaku is credited with the first application of immobilised enzymes in 1969. It used aminoacylase carried on DEAE-Sephadex for the continuous production of L-amino acids from acyl-DL-amino acids, reducing overall operating costs compared with a free-enzyme, batch process by 40%.

Now five companies are in active production, in processes involving four different fixed enzymes — aminoacylase, aspartase, glucose isomerase, and penicillin acylase — and three fixed cells — *Escherichia coli*, using its aspartase metabolism, *Brevibacterium moniagenes* (fumarase) and *Streptomyces* sp. (glucose isomerase). The Tanabe Seiyaku company has also developed processes involving fixed cells of *Pseudomonas putida* (L-arginine deiminase), *Achromobacter liquidum* (L-histidine ammoniolyase), and *E. coli* (penicillin amidase). Many biotechnologists believe that the fixation of cells and cell organelles is the way forward for enzyme technology, and perhaps also

for the use of genetically engineered micro-organisms.

Japan's industrial interest has to some extent been stimulated by the numerous traditional fermented food industries, producing miso and shoyu and the drink sake through the *Aspergillus oryzae* amylases. The average consumption of miso is apparently 24 gm/person/day, and the producers have not been slow to attempt to capitalise on the new biology. The fermentation industry represents some 2-3% of Japan's GNP.

Outside industry, government has also taken a strong interest, particularly through the Office for Life Science Promotion headed by Professor A Wada. This office has set up a systematic programme for the research and development of enzyme technology, and has made a detailed analysis of its possible impacts on industry and society. "The association between a well-defined plan and the Japanese potential is unique in the world" writes Thomas in his report.\*

The recently published French report\* suggests that Japan has chosen to make an all-out effort on biotechnology because of the potentially high added value the technology can give to imported raw materials. In a country as dependent on imports as Japan this is an important consideration.

Furthermore Japan already enjoys "a very high reputation as one of the principal world producers of antibiotics" through fermentation, and the country intends to maintain this position through its research. "It is clear that in Japan there is no official

or conceptual division between basic and applied research; there is a continuous exchange of skills and services between universities and industry".

However there is a feeling that Japan is not as advanced as some other countries in fundamental biology, so international competition particularly through genetically engineered organisms is not closed.

## US catches up

America is the second most advanced country in this field, judging by patents and production. "The patent list shows that industrial interest in enzyme technology is significant, but so far the only large scale application is the production of fructose syrups by several companies" writes Thomas.\*

But he forecasts an "explosion" in applications which should flow from the Research Applied to National Needs programme of the National Science Foundation. A strong interdisciplinary team at the MIT has been focusing on the total enzyme synthesis of gramicidin, a cyclic decapeptide antibiotic. This problem tackles head on the major obstacle to application of synthetic as opposed to degradative pathways: the cost-effective provision and regeneration of small co-factor molecules such as ATP and NADH.

The country that finds a practical solution to this problem, and patents it, will find itself at a major advantage compared with others; but there is some scepticism about whether extra-cellular systems will ever be successful.

Some eight cell species have been successfully immobilised in the laboratory in the US: *Streptomyces venezuelae* and *Bacillus* sp (for producing fructose), *Saccharomyces cerevisiae* (invert sugar), *E. coli* (fumaric acid), *Nocardia erythropolis* ( $\Delta^4$ -cholesterone), *Corynebacterium simplex* (prednisolone), *Serratia marcescens* (2-keto gluconic acid), and *Corynebacterium lilium* (glutamic acid).

Moreover the US is well-advanced in applied recombinant DNA techniques — the production of somatostatin for research purposes being promised shortly, with insulin also on the way; and it has a very strong base in fundamental biology. Research and development organisations based on venture capital are very active (see *Nature* 278, 494; 1979), and at least one (Genentech) is active in recruiting scientists outside the US, particularly in Europe.

## Soviet secrecy

Soviet commitment to biotechnology, indeed to all aspects of molecular biology, began officially with the decree of 21 May, 1974, which set Soviet science the task of "accelerating the development of molecular biology and molecular genetics" and the "wide use of their achievements in agriculture, medicine and industry". Considerable human and financial resources were committed to closing the

\*"Genetic manipulations in applied biology" by A Rörsch, catalogue number CD-NI-78-001-EN-C; "Production of biological catalysts, stabilization and exploitation" by D Thomas, CD-NI-78-002-EN-C, available from offices for official publications of the European Communities; and "Sciences de la vie et Société" by François Gros, François Jacob, and Pierre Royer, available from La Documentation française.



gap left by Lysenkoism.

Partly as a result of Lysenkoism, Soviet "applied" genetics has a strong commitment to radiation-produced mutations. However, several groups throughout the USSR are known to be working on applied genetic engineering, and there have been several hints that the industrial breakthrough point for "certain medical products" made in this way will be reached "in the nearest future".

Most socialist countries, however, are somewhat secretive as to detail. One exception is Hungary, with its international biology centre at Szeged (built largely by UNDP funds). Work at Szeged, however, is still largely at the stage of establishing techniques and principles. One team, researching on nitrogen fixation, recently started work on establishing a genetic map for *Rhizobium* and studying the genetic structure of chemically produced mutants.

In Poland, however, the Institute of Biochemistry and Biophysics of the Academy of Sciences would put the breakthrough point for nitrogen fixation by "agricultural plants" at the order of 20 years ahead. According to interviews which specialists from the institute recently

gave the prestigious weekly *Polityka*, they would put on the same time-scale the modification of cereal genes so that the grain proteins would provide the whole range of aminoacids needed for human nutrition. In the "near future", on the other hand, they expect the commercial production of hormones (unspecified), vaccines and special microorganisms designed to clean up pollution. **Vera Rich**

## Germany leads in Europe

A major initiative in the European Economic community is discussed opposite — but, in outline, Europe starts well behind Japan and the US in the application of modern biology. Within Europe, West Germany is generally reckoned to be in the lead: government spending on the basic problems of biotechnology is about ten times that in France or the UK, for example. Altogether, between 1972-78, the federal government invested £35 million in biotechnological R&D.

One of the highlights of the German effort is the Gesellschaft für Biotechnologische für Forschung mbH at

Braunschweig near Hannover, with an annual budget in 1977 of some £5 million. It was created in the early days of biotechnology — the mid sixties — by the Volkswagen foundation, but was later taken over by government. It has a staff of some 80 researchers and support staff, but has been criticised by some for being insufficiently productive. A new director, Professor Kieslich, has recently been appointed.

The science ministry (Bundesministerium für Forschung und Technologie) also plays an active part in funding and planning biotechnological research and development in university and industry; its programme is the envy of biotechnologists in other European countries. So far Germany's greatest efforts lie in its government based research programme.

Italy has a fairly advanced programme, particularly through the activities of the firm SNAM-Progetti, which operates a large laboratory devoted to biotechnology at Monterotondo, near Rome; and Sweden can boast ground-breaking work at the Universities of Lund and Upsalla. In Switzerland Nestlé "is devoting a big effort" (Thomas). (UK and French programmes discussed opposite). □

# The origins of biotechnology

THE most ancient biotechnological art is fermentation — in the raising of dough, the brewing of alcohol, and the production of tea, coffee, and cocoa (each of which requires a fermentation step). But the new wave of biotechnology rests on two main techniques: genetic manipulation and enzyme (or cell and cell organelle) immobilisation.

Genetic manipulation is used by the biotechnologist to enhance the natural genetic repertoire of a microorganism. The aim is usually to donate to the microorganism a gene for an enzyme or a hormone in such a way that the product of that gene is thenceforth synthesised by the microorganism. The successful development of genetic manipulation has rested upon the discovery of new enzymes and the improvement of old techniques by molecular biologists and microbiologists.

Crucial to the success has been the development of vectors for carrying foreign genes into microorganisms. Also of great importance has been the acceleration of DNA sequencing technology.

The real cornerstone of genetic manipulation, however, has been the discovery of enzymes that cut DNA at specific sites (restriction enzymes), seal the cuts up (ligase) and copy DNA from messenger RNA (reverse transcriptase). The first two of these are needed for the insertion of foreign genes into the DNA of the vector. Reverse transcriptase has become particularly important in the last two years since the revelation that the genes of higher organisms are 'split' (the coding DNA

being interrupted by non-coding segments). In sharp contrast, bacteria do not have split genes and cannot decode split genes that are transferred to them. Therefore it is necessary to transfer an unsplit equivalent of the gene, which is precisely what reverse transcriptase produces from messenger RNA. Alternatively, due to recent advances in the technique of DNA synthesis, it is also possible to make purely synthetic genes at least for small peptide hormones.

The use of enzymes is an older technique, applied in a number of industries from baking to detergents. But purified enzymes are soluble molecules and it is therefore not easy to separate them from the product of their labour. Furthermore it is difficult to recycle the enzyme. These difficulties have led to the development, in the late 1960s, of immobilised enzymes. The enzyme is bound chemically to a solid matrix or entrapped in a small-pore gel.

Immobilised enzymes have been successfully applied, for example, to the production of semi-synthetic penicillins, to the large-scale production of fructose from maize, and to a simple assay for blood cholesterol.

Immobilised cells or organelles have potential advantages when the stabilisation of an enzyme is difficult, or when a coenzyme or sequence of enzymes is required for synthesis.

Biotechnology may be dominated by microbial and enzyme technology, but it is certainly not synonymous with them. Both

animal and plant cells have their place in the armoury of the catholic biotechnologist. Successful exploitation of animal cells has been achieved, for example, by Wellcome Research Laboratories in the production of interferon from virus-stimulated lymphoblastoid cells.

A completely distinct exploitation of animal cells lies behind the commercial production of high grade antibodies for research, diagnostic and clinical use (for example for tissue typing prior to transplantation). The key to that is the hybridoma cell, an 'invention' of five years ago. Natural antibody-secreting cells neither survive long enough in culture nor produce pure enough antibodies to be a commercial source of antibody. But a hybridoma cell, the result of fusing an antibody-producing cell with a tumour cell, grows and divides continually. And all the cells from the original hybridoma secrete the same, pure antibody.

Work is also going ahead in many countries on the problem of nitrogen fixation — attempting to extend the range of associations of the nodule-forming, nitrogen-fixing, bacterium *Rhizobium* to plants other than the *Leguminosae* (particularly cereals), or to transfer the nitrogen fixing genes to plants — a distant prospect, given the extreme oxygen sensitivity of the pathway. There is also the distant prospect, through genetic manipulation, of a new source of controlled genetic variability in plants to produce new high yielding or resistant varieties.

**Peter Newmark**



# Brussels asked to spend £16m on biotechnology

Today, the EEC's thirteen Commissioners are being presented with a proposal for a five year (1981-85) community programme for research and development in 'biomolecular engineering'. Here, **Robert Walgate** outlines the proposal and the rationale behind it. Over the page, he summarises a report on biotechnical safety prepared for the Commission.

THE proposal being put before the Commission has been originated by a small team of officials in the research directorate of the Commission; the officials are themselves active in biological research, but their proposal is based on a wide range of advice from experts, government officials, and industry received over the last two or three years. It will only become official if first, it is approved by the Commission and second, it is accepted by member governments through the Council of Ministers. If all went smoothly, this process could be complete by the end of April.

The programme would be an 'indirect action', whereby the Community budget would contribute half the costs of research projects, and contracting parties the other half. The proposed Community contribution is 23.5 million European Units of Account (about £16 million) divided over nine countries and five years — crudely some £300,000 per year per country, although the allocation will be strictly according to the quality and suitability of individual applications. The amount is small compared to Germany's

national effort, but would in the UK for example, match the Science Research Council's present expenditure — so the proposal is far from negligible. European industry has recommended its speedy implementation.

There would be six projects in the programme. They are designed in part to tackle basic technical obstacles to the development of biotechnology, and in part to suit particular European problems. The programme is outlined below.

## French views

Professor Daniel Thomas, enzyme technologist at the new University of Compiègne, near Paris, is naturally in favour of an EEC effort: he wrote the report which forms the basis of the first and second projects. He feels one of the main advantages of an EEC action will be that it promotes cooperation. 'In Europe we have better contact with the US than we do with each other' he says. Cooperation might be difficult on development questions, but on basic research a coordinated programme would be 'very helpful'.

'There are some 4000 PhD

biotechnologists in Japan; in France we have only about 200. We need a critical mass in Europe to compete with Japan and the US.'

Fundamental biology is of a very high quality in Europe, Thomas believes, and though industry is at a low level, it is good enough to succeed 'if there is a big effort'. But Europe cannot hope to 'reproduce Japan'. The first ten companies in biotechnology in Japan have grown out of the food industry, which took an early enlightened attitude to the new biology.

In America, dedicated contract research organisations such as Biogen, Cetus, and Genentech were thought to be the answer 'but it would be better in Europe to arrange loose links between academic institutions and industry'. This is one of the objectives of the proposed EEC programme.

A big leap forward in biotechnology requires 'both high level work and classical fermentation chemistry' says Thomas. The effective application of genetic manipulation is, he believes, a long term goal; 'in the short and middle term we must look at enzymes and fermentation'. The fundamental bottlenecks in enzyme technology could be solved — scientifically at least — within five years, he believes.

France has been slow to recognise the importance of biotechnology. 'Before the report to the President (see footnote p123) the very words 'biotechnology' and 'enzyme technology' did not exist in

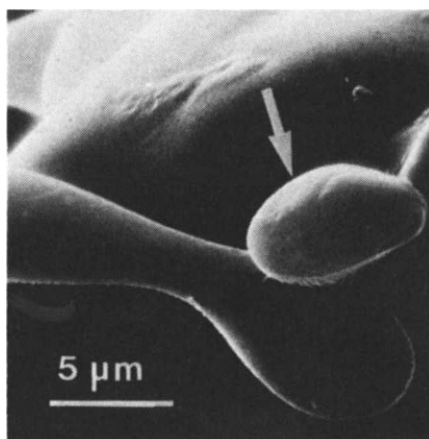
## The proposed EEC programme

In bare outline, the programme consists of:  
**First project:** enzyme technology, involving

- reactor kinetics and geometries for multi-enzyme reactions
- immobilisation of multi-enzymes and co-factor regeneration
- stabilisation of lipid-requiring enzymes and development of reactors for reactions at a hydro-organic interface
- stabilisation of sub-cellular organelles such as mitochondria, chloroplasts and peroxisomes and of membrane-bound enzymatic complexes in the form of microsomes, thylakoids, and inner mitochondrial and plasma membranes; and of intact eukaryotic plant and yeast cells.

**Second project:** development of methods for industrial and human detoxification, involving

- bio-compatible insoluble matrices
- targeted carriers, with specific tropisms, for administering medical enzyme products



*Immobilised lettuce chloroplast: the EEC's energy programme is already supporting research on the production of hydrogen from immobilised chloroplasts associated to bacteria hydrogenase*

- enzymatic detoxification of poisons.

**Third project:** transfer of genes to *E. coli*, the yeast *Saccharomyces cerevisiae*, and 'other suitable organisms', involving:

- overcoming expression barriers
- construction of 'synthetic genes'
- development of mutational tools to produce, for example, harmless vaccines

or stabilised enzymes

- modification of enzymes to inhibit their degradation in a foreign environment
- development of 'libraries' of expressed genetic information and rapid screening techniques for their identification

**Fourth project:** development of cloning vehicles in organisms which are likely to be of greater importance to industry than *E. coli*, such as the bacteria *Actinomyces*, *Pseudomonas*, and *Bacilli*, the fungi *Penicillium*, and algae, using plasmids, viruses, and mitochondrial DNA; and in plants using potential vehicles such as *Agrobacterium* plasmids, plant DNA viruses chloroplasts and mitochondria.

**Fifth project:** stability, regulation, and expression of transferred genes, including the regeneration *in vitro* of plant cells

**Sixth project:** improved methods for detecting contamination in fermented strains.

In each of the six projects, some three to 12 research groups would participate; each group would consist of, on average, three researchers, two technicians, one laboratory helper, and 'secretarial assistance'.

France" says Thomas. Funds for his work at Compiègne came in small amounts from a dozen different sources. But now, after saturation coverage for two weeks on television and the newspapers "everyone was trying to do it".

But there are dangers in this sudden enthusiasm, he feels: biotechnology is not the panacea the media have taken it to be.

### English disease

Looking over the water to England, Thomas is "very surprised" that the University College, London, group of Malcolm Lilly and Peter Dunhill — the first in the field — get so little money".

Lilly, it should be said, does not complain at the size of his share of the small British cake in biotechnology, but is unhappy with the size of his group: "two more lectureships and an administrator would change the picture overnight" he told *Nature*.

Lilly's group is unique in the UK in combining chemical engineers and biologists — essential for good biotechnical work, Lilly believes. Biologists must feed the engineers with science, and the engineers feed the biologists with problems; and "ideas are transferred from person to person". Technology transfer is, Lilly feels, "bloody hard work!".

He spends about one day a week talking with industrialists: "one desperately wants good industrial/academic liaison".

There are two categories of biotechnologist in England: the recognised specialists, and people in associated fields

who are attempting to find application for their work. "These people need guidance" says Lilly.

There are very many plausible applications, "but we have learned to be very selective in what we offer industry". Much work needs to be done between idea and application, and an ill-thought out idea will ultimately fail.

The number and age of biotechnologists is a central problem in the UK, he believes. "Most specialists are in their forties, and isolated in biochemical or engineering departments" where they have risked becoming black sheep. Very few departments have appointed younger people to follow them through. "The reason we've done better than others in the UK is that there were always two of us. We could talk to each other."

Lilly's group now consists of three full-time researchers, plus 20 technical staff and research students. "If I had two vacant lectureships I could fill them tomorrow" he says.

The potential economic productivity of such an investment is high, he believes. In the early days of enzyme technology in the UK some £500,000 was spent on research grants. The result was industrial immobilised enzyme processes, of which the biggest was fructose production, then the deacylation of benzyl penicillin.

For the future the main problem is handling co-factors in biosynthetic pathways. But here one should not distinguish enzyme and cell immobilisation: for example with glucose isomerase for fructose production some

firms use cells, others the isolated enzyme. Where co-factors are required, whole cells have the edge. Fermentation also will remain important, particularly for methane production and waste treatment.

The key to industrial success, Lilly believes, will be to look for products of high added value, insulated from volatile market forces. For instance, the fermentation of sugar to produce industrial alcohol "is at the whim of the weather", which affects sugar prices; and single cell protein is similarly affected by the price of conventionally grown soya protein with which it competes as fodder.

Professor Lilly may take heart from the signs that the UK is about to make a second effort in biotechnology: a Royal Society committee on biotechnology is about to report, there are moves afoot to set up a British version of the American Biogen company, and the National Research Development Corporation is reportedly poised to make an increased investment — more in proportion to its revenue from biological patents, which greatly exceed its revenue from physical technologies.

And at the Science Research Council, which currently spends some £300,000 a year on biotechnology, a joint panel is shortly to be set up involving industrialists and academics; the panel will recommend by the Autumn a coordinated programme in biotechnology. According to Dr Geoffrey Potter, chief administrator of the SRC's Engineering Board, one of the main objectives will be to train new biotechnologists, or to help existing biologists shift towards technology. □

## How safe will biobusiness be?

"THE COW," write two biologists invited by the European Commission to produce a report on the hazards of biotechnology, "is a live, mobile, self-reproducing and edible fermenter." To digest grass and produce milk it employs a complex and varying mixed culture of anaerobic micro-organisms in its rumen; some of these have not even been characterised.

"The cow can become infected with pathogens, including foot and mouth disease virus, *Brucella abortus*, and *Mycobacterium tuberculosis*, which can damage or destroy the fermenter and infect the product. In the past it has been a major source of human brucellosis and tuberculosis.

"A singular disadvantage of this fermenter system is that it can only be scaled up by increasing the number of units; moreover its productivity, in terms of protein production, is only 0.0025 compared with that of an equal weight of microorganisms growing in an industrial fermenter."

Conjectural hazards of supposedly 'unnatural' techniques are not of happy memory for molecular biologists who alerted the world to what are now widely

accepted as the largely imaginary risks of research using recombinant DNA. But painful though it may be, the questions must be re-thought if industries based on these and other new biological techniques are to be set up.

The scale of industrial processes, using 1000 to 30,000 litre fermenters, is vastly greater, and process control is more difficult. Also there is an economic disincentive to using severely disabled strains, and little attention has been paid to the dangers of the use of plant pathogens.

These and other issues have been raised in the report quoted above by Drs K. Sargeant and C.G.T. Evans of the Microbiological Research Establishment, Porton, UK. They conclude that:

- The special hazards of biobusiness rest only in the potential of some micro-organisms to infect higher species and cause disease.

- "With the exception of a few that affect plants and insects, it is very unlikely that dangerous pathogens will be used on a large industrial scale."

- Research is needed on the long-range airborne transmission of, especially, plant pathogens.

- "For diagnosis, research and vaccine production some highly dangerous human and animal pathogens must continue to be grown on a small scale. A list of such organisms should be drawn up, and their handling, except under licence, should be forbidden. . ."

- "The chemistry of a proved and licensed fermentation process can alter in response to genotypic and phenotypic changes in the culture. These can be difficult to detect, especially in long-term continuous processes."

- Hence, research is needed on the rapid detection of small numbers of contaminant microorganisms within large populations; and on the mechanisms of, and potential for, phenotypic change.

On regulation of the industry in Europe, Sargeant and Evans argue that biotechnology will be "too diverse to be encompassed by a single set of regulations". Rather, they recommend that "regulations should be worked out by associations of manufacturers of product types in conjunction with governments, and harmonised within the EEC. This is already happening for food enzymes and single cell protein".



In their review of hazards, Sargeant and Evans keep the problem firmly in perspective. "Pathogenic micro-organisms are a normal feature of our environment, held in check by Public Health measures and an informed food technology." The dangers of large-scale biotechnology must be seen against this background.

The main new features are scale of production, the large numbers of workers exposed to the organisms and their products, and the possibility of inadvertent release affecting the environment.

'Avirulent strains' may not be what they seem. "Avirulence is a matter of degree, related to dose, portal of entry, and resistance of the host. Although so far unknown, the enhancement of virulence by phage transduction is a possibility. . . that should not be entirely ignored."

The production of diphtheria toxin, the authors point out, is associated with the presence within *Corynebacterium diphtheriae* of a bacteriophage.

Further, the contents of the fermenter may not be what they were thought to be. They may become contaminated with foreign organisms (unless the fermenters are well closed), or there may be phenotypic variation.

One of the main ways the latter could occur is inadvertent change of the growth limiting nutrient, perhaps by failure of the supply line of a medium component. "Cell walls of rapidly growing phosphate-limited *Klebsiella aerogenes* NCTC 418 are only slightly toxic to mice whereas similar, but carbon limited cell walls are highly toxic."

The change from batch to continuous culture can also change the character of an organism: batch grown cells of the live vaccine strain of *Francisella tularensis* are much more toxic to mice than the continuous grown cells, although the protection is equal.

Change of temperature can also be important. At 15°C and 45°C the aflatoxins are produced by *Aspergillus flavus* in only trace amounts on groundnuts; at 35°C, under otherwise identical

conditions, 40mg/kg are produced. And change of pH through failure of the control system — "a not unknown occurrence" — can also cause major phenotypic changes. Change of trace elements in the feedstocks may also stimulate or inhibit the production of particular metabolites.

Sargeant and Evans illustrate the danger with "an entirely hypothetical, but easily credible, example".

"Until 1960, *A. flavus* was regarded as a harmless mould closely related to the *A. oryzae* strains used in Japan for making miso, shoyu and sake. It would not have been unreasonable to think that a pure culture of *A. flavus* could be eaten with impunity.

"In 1960, major outbreaks of a 'new' disease among turkey poult and ducklings — turkey X disease — were encountered in the UK. The cause was traced to a new group of toxins, now known as the aflatoxins, which were generated on contaminated groundnuts by some strains of *A. flavus*.

"It has since been shown that aflatoxins can cause serious disease in many animals, including man; epidemiological studies suggest that they are a major cause of human liver cancer in some parts of the world.

"*A. oryzae* was being developed in 1960 as a source of amylases for bread making, but it could have seemed reasonable to develop *A. flavus* instead. Alternatively a potentially toxigenic strain of *A. oryzae* could have been used, and, by chance, growth conditions that did not allow aflatoxin production could have been chosen.

"After careful testing, the product could have been declared safe. Subsequently, a small inadvertent change in the growth conditions could have led to the production of very heat stable aflatoxins and to their inclusion in our daily bread."

However, those primarily at risk are the workers, subjected to "the dense aerosols of microorganisms which, in the absence of suitable precautions, will be released into the factory atmosphere during recovery of the products of fermentation by centri-

fugation, spray, or flash drying, packaging and handling of the product, and release of the culture supernatant."

Outside the factory residents are no more likely to be at risk than those near a mechanically aerated sewage works "with its potential population of human pathogens".

The products of enzyme-based factories — such as the high fructose syrups used in soft drinks — may be contaminated with enzyme, and the effect of long-term low-level exposure is unknown. But the non-biological contaminants of soft drinks — such as sugar and phosphoric acid — may themselves have greater deleterious effects.

Man's environment could be affected by the discharge of nutrients, increasing microbial, algal and plant growth, but this problem is not exclusive to biotechnology.

The discharge of live organisms might have ecological effects, but since the strains will be unnaturally specialised their release would be "tantamount to abandoning an over-bred and pampered European lap-dog to its fate in Siberia. We have been authoritatively informed that when, some years ago, the contents of a very large antibiotic fermenter were inadvertently discharged into the sea, no trace of the organism could be detected the next day, despite a very thorough, widespread, and anxious search."

More dangerous might be the continuous low-level discharge of antibiotic, creating ideal conditions for the selection of resistant organisms. If contained on a plasmid that information could be transmitted throughout the microbial world. However, the widespread use of antibiotics in animal feedstuffs is a greater danger, the authors suggest.

The use of plant pathogens is increasing: "the 400-litre production scale of the anti-leukaemia enzyme asparaginase from *Erwinia chrysanthemi* — pathogenic for chrysanthemums — has been completely overshadowed by that of xanthan gum from *Xanthomonas campestris* — pathogenic for brassicas."

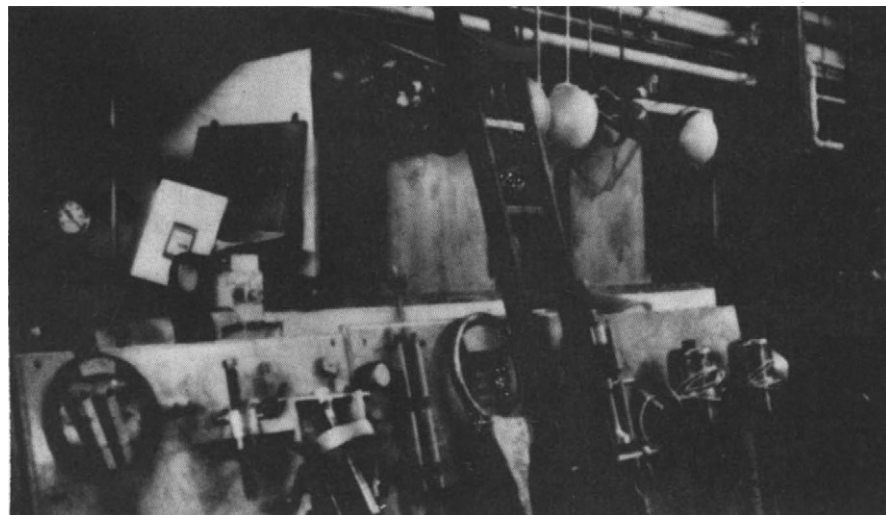
Most serious is the potential airborne dissemination of such organisms to countries in which they are not indigenous; "and their aerosol survival and infectivity have been attributed to the production of the very gum for which they are grown".

Finally there is the danger of the establishment of new genotypes during continuous cultivation, where organisms are maintained through many thousands of generations.

"If a contaminant arose which was able to maintain itself...then no guarantee concerning either the safety of the process or the culture could be given.

"The situation is in this respect fundamentally different from farming with plants and animals, where much more precise control of the genotype is possible — because one generation is cultivated at a time and alterations in the genotype are...much more obvious."

Robert Walgate



Hard-headed about safety: inside a biotechnical laboratory in London

# Patenting living organisms — how to beat the bug-rustlers

FIVE days before Christmas, the Upjohn Company dropped a firecracker from one of the most keenly-awaited decisions facing the Supreme Court — whether patents can be granted for living organisms.

Officials of the pharmaceutical company say they have withdrawn a patent application for a strain of bacteria capable of producing the antibiotic lincosamin, first filed in 1975, to focus the court's attention on a second application involved in the same decision, filed by scientists with the General Electric Company.

Unlike the Upjohn bacterium, which is essentially the refinement of a strain discovered in nature, the GEC case involves a strain of bacteria of the *Pseudomonas* genus genetically altered to maximise its effectiveness in degrading oil spills — a manipulation which, although it did not make use of the now-familiar gene-splicing techniques, is still considered "genetic engineering".

Yet the Upjohn patent application had already been approved by the Court of

Attempts to patent 'live' biological processes have raised wide debate about the legal and philosophical implications. But patent protection may prove less attractive than a strategy of closely-guarded research and swift commercialisation. **David Dickson** reports from Washington

Congress has already faced this dilemma. In the 1920s, when plant breeders began pressing for patent protection for new strains of seeds, it decided that plants were not covered, and in 1930 it passed the Plant Protection Act broadening the definition of patentable matter to include certain varieties of plants.

The argument of those now seeking to patent microorganisms is that, as previously the case with plant biochemistry, modern biology has so blurred the distinction between animate and inanimate matter as to make the concept of life — in the strict legal sense — almost irrelevant.

"Surely where the line between life and non-life is so fine as to baffle even the

by present legislation; that Congress's previous introduction of new laws covering plants was a recognition of this fact; and that given the wide social and ethical implications of the current cases, the issues should be resolved by Congress rather than the courts.

If the Supreme Court decides to pass the buck back to Congress — and this would be consistent with other recent rulings — the stage will be set for a further round of public debate on the social consequences of genetic engineering. And in this case, the focus will not be on their potential hazards, but who is entitled to benefit financially from their use.

Opposing the argument that the creation of substances by live processes should be treated no differently from ordinary mechanical production processes, critics say that sufficient protection for useful techniques can still be obtained without necessarily giving away title to complete organisms.

Dr Burke Zimmerman, previously a congressional staff member closely involved with attempts to legislate recombinant DNA research and now with the National Institutes of Health, believes that a law permitting the patenting of living organisms would lead to "irresolvable logical inconsistencies". "To patent a living organism that is to be used only in a very specific way is *not* equivalent to patenting the particular use one may wish to put it to," says Dr Zimmerman. "Thus the classical justification for patenting an invention or even a composition of matter breaks down when applied to a living organism."

Others go further to argue the moral case as part of a general critique of the undesirability of genetic engineering. It is strongly put by the People's Business Commission, a Washington-based research organisation. "To justify patenting living organisms, those who seek such patents must argue that life has no 'vital' or sacred property; that all of life's properties can ultimately be reduced to the 'physico-chemical,'" says the PBC in a brief filed in support of the Justice Department's appeal to the Supreme Court. Furthermore, says the PBC, any such decision could set a dangerous precedent. "If patents are granted on

## 'The line between life and non-life is so fine as to baffle even the experts'

Customs and Patent Appeals, overturning an earlier rejection by the Patent and Trademark Office. The company's surprise withdrawal is therefore a further indication of the extent to which the recent rapid marriage between modern biology and industrial production has caught traditional legal machinery unawares.

Furthermore, while lawyers and philosophers debate the subtleties raised by the private ownership of living matter, others claim that patent protection may be only marginally relevant. Greater profits can be found in a combination of closely-guarded research and rapid commercialisation of the results, closer to the strategy of advanced electronics firms than the conventional ways of doing business in the chemical or pharmaceutical industry.

Turning scientific knowledge into a commodity has produced relatively few problems of definition in the physical sciences. Biology has proved less amenable. The Appeals Court, in granting the two patent applications, found little difficulty in classifying microorganisms as "industrial tools", or in concluding that "life is largely chemistry" — but critics see it as the thin end of a wedge that opens the door to private ownership and control of the evolutionary process itself.

experts in the art and at times cannot be drawn with conviction, compositions of matter or manufactures near the periphery cannot conveniently be deemed patentable or unpatentable on so ephemeral a ground," according to an *Amicus Curiae* (friend of the court) brief filed by the University of California at Berkeley.

According to such arguments, there is nothing special about life that makes it inherently unpatentable. "We do not see any sound reason for making the distinction between the living and the dead," wrote Judge Rich in giving the Appeals Court's reasons for approving the patent application.

According to the court, it is precisely because organisms are alive that they can be useful. "We see no sound reason to refuse patent protection to the microorganisms themselves, or to pure microorganism cultures — the tools used by chemical manufacturers in the same way as they use chemical elements, compounds and compositions — when they are new and unobvious."

The Solicitor General, in appealing the decision to the Supreme Court, has sidestepped the philosophical issues to argue that, whether or not living forms should be patentable, they are not covered



microorganisms there is no scientific or legally viable definition of 'life' that will preclude extending patents to higher forms of life," the group argues.

Yet whether the issue is settled by the Supreme Court or by Congress, many now argue that the patent system may be incapable of providing adequate protection. Although patents provide 17 years protected use, they require in return full publication of the procedures used as a form of public education. Many fear such disclosure could provide important clues to potential competitors who, without directly infringing patent rights, might quickly come up with rival products. Others argue that although an application may be granted by an examiner, it may prove difficult to defend because of the general ubiquity of biological research.

As an alternative (or complementary) strategy, many companies now rely primarily on research carried out in relative secrecy backed up by intense marketing once the results have reached a saleable form. One executive speaks spiritedly of having to guard against "bug-rustling"; and speed and flexibility, rather than legalised monopoly, have become the keys to success.

"Opinion seems to vary across the spectrum, from those who feel that the patent issue is the most important thing we face, to others who feel it is almost irrelevant", says Nelson Schneider of investment analysts E.F. Hutton, a close observer of biotechnology developments. "The problems are more in the area of fears about what someone else is going to do, rather than seeing anyone else do it. I am aware of no-one who, if they made a really great breakthrough in the field, would immediately file a patent application — they would prefer to sit on it."

## **'The classical justification breaks down when applied to a living organism'**

That market dominance can be as effective as patent protection has already been illustrated by the pharmaceutical company Eli Lilly. Last year Lilly, which currently has about 85% of the domestic insulin market, agreed with the Federal Trade Commission to make both supplies of animal pancreas and the appropriate refining technology available to anyone interested — yet such is the company's prominent position in the market, and the economies of scale it is therefore able to effect, that other companies have been reluctant to take up the challenge.

Eli Lilly is now engaged with Genentech in developing the artificial production of human insulin, with hopes that it could be on the market by the end of the year. And this speed is dictated partly by the need to

## **'... a philosophical reductionism already responsible for the destruction of natural ecosystems and alienation of humanity from nature'**

head off potential competitors who may also be working on synthetic insulin production.

A heavy emphasis on marketing may be even more necessary to maximise returns on another new biotechnology which is expanding rapidly, the production of monoclonal antibodies with highly specific characteristics from hybrid cell lines. By fusing a mouse spleen cell and a rapidly-reproducing tumour cell, and selecting out those resultant cells producing antibodies with particular desired characteristics, Dr Cesar Milstein and Dr George Kohler at Cambridge, England, have opened the way to a reliable method for the general production of antibodies.

But even more than with genetically-manipulated microorganisms, the information contained in a patent application on a hybrid cell-line describing its usefulness may provide an idea of how the same result could be achieved by a slightly different route. "Even if it does turn out to be possible to patent cell lines, we are not sure that it would be worthwhile," says Dr Howard Birndorf, vice-president of Hybritech, a west coast firm set up with the same source of venture capital as Cetus and Genentech. It is already marketing monoclonal antibodies to the hepatitis B surface antigen, and promises many more. "Rather than going for patent protection, we have chosen to develop techniques and processes in-house, staying more under trade-secret type protection," says Dr Birndorf.

Once a technique is perfected, the strategy is to market the product as quickly and as widely as possible, attempting to build a quick lead over potential rivals. "In immunodiagnostics the person who is first traditionally gets the largest share of the market," says Dr Birndorf. And he compares the hybridoma field to the semiconductor industry, where it is the company that can raise the number of useable silicon chips from two to 30 in a hundred that wins out.

But while secretive research and aggressive marketing may suit the style of the small, venture-capital-based firms, they fit less easily into the traditional *modus operandi* of biological research.

Many biologists, caught up for the first time in what may turn out to be highly lucrative research, but uncertain of procedures and frightened of giving anything away unwittingly, have become confused about what to do. Others are accused of violating the spirit of the research community.

"Scientific meetings in this field used to be free and open," says one observer. "Now everyone sits on their hands in a corner, not talking to anyone else. There is

a fear of what you may give away — and how long it may take before you know what you have done."

Mr Neils Reimers, head of the Technology Licensing Office at Stanford University, claims this is as much the fault of the rush to publication as of the demands of the patent system. But he accepts that arranging the commercial exploitation of biological advances in ways mutually beneficial to the public at large, private corporations and the university scientists who carried out the original work had raised problems.

Even with the recombinant DNA techniques, once a patent has been granted, it may prove difficult to enforce. Stanford, for example, has already applied for a share of the patent rights to the original gene-splicing techniques developed by Dr Stanley Cohen and Dr Herbert Boyer. And if this is granted, various companies have suggested that they may challenge the patent if they feel the university is demanding too high a licence fee.

Such difficulties in enforcing patent claims mean that, despite the potentially lucrative discoveries now emerging from biology laboratories, universities may not find them the gold-mine that some have speculated. But there are already moves in Washington to assist universities if they do come up with a winner.

In a package of recommendations issued in November, and designed to stimulate industrial innovation, President Carter proposed among other things a revision of the patent law making it easier for universities and small businesses to retain the patent rights on discoveries resulting from federally-sponsored research.

This will be done through a system of what are known as Institutional Patent Agreements. To qualify for an IPA, a university must demonstrate an ability to transfer research results to the private sector, and must agree to use any income for research or teaching. In return it will be allowed to arrange the licensing of the patented results with a minimum of government interference.

Many current complaints about the patent system are addressed in a bill passed by the Senate last autumn, and sponsored by senators Birch Bayh and Robert Dole. If passed by the House, this will formalise a university's right to profit from the discoveries of its scientists in a manner similar to the Administration's proposals.

Others, however, are worried that the corporatisation of university research, as it stretches down to the workings of the cell, may reinforce a philosophical reductionism already responsible, it is claimed, for the destruction of natural ecosystems and the increasing alienation of humanity from nature. □

# The ties that bind or benefit

As industrial corporations become more involved in developing new biological techniques, where does this leave the scientist? How will university biology departments maintain their integrity and autonomy? How will individual scientists react to growing corporate demands? These and other questions were explored when *Nature* brought together **Sheldon Krimsky**, Acting Director of the Program in Urban, Social and Environmental Policy at Tufts University, and **David Baltimore**, Professor of Biology at MIT.

**Krimsky:** I am very concerned about the growing influence of industry on academic science. This has existed for many years in the chemical and the nuclear industries. And now it is beginning to occur in the biological sciences, especially with the advent of advances in recombinant DNA.

The direction of research influenced by industry may not be the direction that is in the public interest. Corporate requirements for scientific research may be at odds with pressing societal needs. There are many examples one could cite, especially in environmental research, nutrition and biomedical science.

Our academic institutions and academic scientists need to be free from corporate influence. And such influence can emerge in various ways: through direct funding by corporations to departments and investigators, by scientists serving as consultants to or on advisory boards of industrial firms, or by academic scientists playing a double role by setting up their own small venture-capital firms.

These are dangerous moves if they become widespread because in the end university-based science will lose its sense of detachment. When confronted with policy decisions on the applications of technologies to society we need objective academic involvement. How can the public have confidence in the expert opinion of scientists on the potential risks and benefits of a technology when those same individuals have financial interests in commercial development?

**Baltimore:** It seems to me that there is nothing wrong with universities serving as a source of technical expertise for newly developing industries or even for mature industries. And that kind of symbiosis between industry and academic departments has been very good for both over history — take the chemical industry.

This seems to be a healthy development for a number of reasons. One is that if there is an industrial use for modern biological techniques, it means that those who are trained in university biological laboratories will have an increased range of employment opportunities. With the recent plateau of hiring into academic positions, it is necessary, if we are going to maintain graduate student activity, to find other opportunities for our graduate students.

What's more, we find that after the large investment in basic biological research we are now beginning to get practical spin-offs. While society may value the advancement of abstract knowledge, that is not society's major interest. Consequently, I find it heartening that we can now say to the public, "You've been investing all this money for so long and now things are emerging that are of practical value."

I, too, worry about the academic enterprise being undermined by corporate interests. We can have both an academic focus and industrial involvement but we must maintain a separation between the two. To make certain that university biology departments maintain their autonomy we must be vigilant about a number of things:

- We must be entirely open about industrial affiliations.

- We must maintain a separation between university and industry. When academics consult for industry to help find solutions to industry's problems, they ought to remain consultants and not turn their laboratories into factories for the solution of corporate problems. To be independent, university laboratories must be allowed to follow the internal logic of science.

- We should not use our students for our own ends. Students should continue to have freedom to work out problems that emerge in the laboratory, without being tied to predefined industrial needs.

If we can handle this mixed economy with honesty and integrity, we will have done a service to the university by bringing in new sources of funding and by increasing the importance of the university to industry.

**Krimsky:** In the 1960s, many scientists (including yourself) expressed moral outrage in learning of the war-related

research carried on secretly at many universities. Now we must ask ourselves what the consequences will be if industries ostensibly fund entire departments (as they have done in chemistry, computer sciences, engineering and, more recently, certain areas of policy analysis). Notwithstanding the fact that corporations claim they give money with no strings attached, we can expect first that they will establish the broad boundaries of research. And second, there will exist a "chilling effect," particularly when industry-funded faculties are inclined to work on projects or express views that can be interpreted as disadvantageous to the goals of their corporate benefactor. Just as war-related academic research compromised a generation of scientists, we must anticipate a similar demise in scientific integrity when corporate funds have an undue influence over academic research.

Crucially, during periods when we worry about the risks of technology, we need independent scientists willing to speak out. Risks may crop up in research itself or in the ultimate marketing of biological products. As you know, in the petrochemical industry we have had very few scientists willing to speak out against the potential hazards of petrochemical agents to workers, consumers or military personnel. And now we are faced with the results of their timidity. With so many scientists working directly from the chemical industry, can we expect them to speak freely about the toxic effects of chemicals? How extensive is the influence of industry on biology going to be?

**Baltimore:** I couldn't agree more. I think it is extremely important that the academic enterprise maintain its freedom to do what it wants, to say what it wants, to be counter to conventional wisdom and to discover things that are counter to the desires of industry.

Nevertheless, the positive side of industrial involvement in the academic world should not be discarded out of fear that university-based scientists will lose their autonomy owing to industrial pressure. We need to maintain a balance between industrial influences and influences from outside the corporate world.

**Krimsky:** Are there any mechanisms



**Krimsky:**  
'Just as war-related research compromised a generation of scientists, we must anticipate a demise in scientific integrity when corporate funds have an undue influence over academic research'



available to universities guaranteeing that balance? And as for spin-offs: what role does society play in determining what the research priorities or the practical applications of biological research ought to be? We can have many different kinds of potential spin-offs. Shall we simply allow corporate management to decide autonomously what path to take? Scientists may feel free to choose the fine-structured problems in a research programme at the same time that the shape of the outer boundaries of that programme are exogenously determined. It is in this important respect that science does not develop from some internal logic. I want to see more public and less corporate influence in establishing the outer boundaries.

**Baltimore:** In the United States the answer to your question is that the desire of people to seek profits determines what research gets turned into useful products for the general economy. If they are not even seen as potentially profitable, they are not pursued or produced. One can argue persuasively that that is not a sufficient reason and that government ought to play a larger development role.

Behind these kinds of disputes stands a naive model: at one side there is a scientific sector with people doing basic research. On the other, independent of basic research scientists, we have the industrial sector, selecting those areas for practical exploitation and deciding how they are to be applied. There is a much more important, neglected relationship. The shape of the outer boundaries of basic research is not merely influenced by the corporate sector alone, either directly or through government, but emerges from research itself. I see the world-wide research effort as a continuum — from the problems determined by the inner logic of science to the problems determined by outside influence.

Many scientists work totally out of the logic of their research as it evolves in front of them. For them it doesn't matter what the rest of society thinks about the utility of what they are doing. These are the projects that in the end often turn out to produce the real surprises. No corporation can control them because no-one can see where they are heading.

At the other end of the continuum there are people doing contract research who have been asked to solve specific problems that industry or government wants solved.

Some research is a mixture: scientists get a little money to do something, trying perhaps to cure a disease and at the same time they discover new phenomena. They then have the freedom to explore these new areas.

**Krimsky:** How are we going to maintain a sufficient number of biologists not tied to the industrial sector, willing to stand up and say: "Wait a minute I am not very confident"? Remember it didn't happen in the petrochemical industry.

## Baltimore: 'The positive side of industrial involvement in the academic world should not be discarded out of fear that scientists will lose their autonomy owing to industrial pressure'



**Baltimore:** Yes, there ought to have been much more questioning of what was going on. Universities should have opened up the debate. The rush of industrial development following World War II undercut the critical function of universities.

We now should look at the strengths of what has happened in the last five to ten years. The environmental movement has been a significant success in its own terms, even though it has not accomplished everything it wanted to. It never will. Nonetheless, it has moved from nowhere to become a major force in our society. That has happened because it has been supported by large groups of people.

**Krimsky:** But what about the scientific community?

**Baltimore:** In fact, many of the leaders in these movements have been scientists. Matthew Meselson, for example, is one of the best-recognised scientists in the academic world and yet he is the person who became most identified with opposing defoliation in Vietnam. The movement to stop biological warfare research (which even I had something to do with) came from inside the scientific community, again led by Dr Meselson. The movement to inhibit the development of recombinant DNA research (which, to my mind, got out of hand) came from within the scientific community.

Or consider Bruce Ames, the one person who has really brought a new dimension to our thinking about environmental dangers associated with cancer. He is one of the most eminent scientists in the United States. The Ames test revolutionises our understanding of the dangers inherent in everyday life. This seems to me to be most responsible action, coming directly from the scientific community. Now in fact industry uses the Ames test widely.

**Krimsky:** But industry has systematically opposed the Ames test as an instrument for establishing policies regarding potential human chemical carcinogens.

What's more, industry has set up its own cadre of scientists and experts to legitimate its opposition to effective testing. The American Industrial Health Council, the American Tobacco Institute and the Chemical Manufacturing Association have all set up private institutes designed to defend their positions and I'm worried that the influence of these institutes will spread into the biological departments of universities.

We do not have sufficient checks and balances. We cannot continue to allow university departments and individual scientists to exercise their own judgements because large amounts of money are likely to be derived from consultancies and from profits from these new biological technologies.

What we need is an independent source of funding, encouraging research on the dangers which might emerge from the new biological technologies. We need to secure the university against undue influence by industry in this new field. There must be a balance between research on developing new technologies and research on the potentially adverse effects of these developments.

**Baltimore:** I agree that we need to strongly counter attempts by industry to undercut legitimate, validated testing.

**Krimsky:** One way to accomplish this would be to give incentives to industry to fund universities indirectly through government agencies. Let the government funding agency distribute support to universities rather than allowing corporations to provide direct funding to departments of biology.

But why should industry do this? Clearly, corporations want to be able to direct research at universities and control the purse-strings.

If such funding went through agencies like the National Science Foundation or the National Institutes of Health, peer review committees could distribute the funding to appropriate university-based laboratories. The public would then have some way of overseeing the way the money was spent. We would then not end up with a closed, insulated system.

**Baltimore:** Where industry directly supports research in a given laboratory, that fact should be on record. One can then see in context the views of a scientist funded by industry when that research worker participates in discussions of work funded by the supporting corporation.

**Krimsky:** Given that scientists play multiple roles — for example there are those who are faculty members at universities, who also consult for industry and, in addition, sit on government advisory panels — shouldn't these multiple roles be on the public record?

**Baltimore:** To the extent that it does not unreasonably interfere with a person's private life, I think it ought to be available information. □

## NEWS AND VIEWS

## Small RNAs and splicing

from Richard Roberts

THE small RNAs found in the nuclei of eukaryotic cells (snRNAs) were first described by Weinberg and Penman more than 10 years ago. They range in size from 90 to 220 nucleotides, are only found in the nucleus and have unusual 5'-terminal structures. These structures resemble the 'cap' structures of eukaryotic mRNAs but contain 2,2,7-trimethylguanosine rather than 7-methylguanosine as the blocking residue. They also contain some additional modified bases notably pseudouridine and 2'-O-methyl nucleosides. Although several (U1, U2, UU3B) have been sequenced by Busch and his colleagues, their function is unknown. This may soon change however as a new experimental attack has been launched which could lead to fresh insights into both the function of these snRNAs and the processing of initial RNA transcripts.

Experiments described in two recent papers (Lerner & Steitz, *Proc. natn. Acad. Sci. U.S.A.* **76**, 5495; 1979 and Lerner *et al.*, this issue of *Nature* page 220) have uncovered an interesting class of subnuclear particles and provide the basis for some fascinating speculation about 'RNA splicing.' The experiments are based on old reports (Mattioli & Reichlin *J. Immunol.* **107**, 1231; 1971; Northway & Tan, *Clin. Immunol. Immunopathol.* **1**, 140; 1972) that certain patients suffering from systemic lupus erythematosus possess circulating antibodies directed against a ribonucleoprotein complex present in the nuclei of many eukaryotic cells. In the first paper, Lerner and Steitz have characterised the RNA and protein components of this complex. They show that the complex contains a specific set of seven polypeptides with molecular weights between 12,000 and 35,000, which are neither histones nor the polypeptides associated with the 30S hnRNP particles. The RNA components are the small nuclear RNAs described above. Different antisera precipitate different subsets of these small RNAs, but always with the same set of seven polypeptides. Furthermore, the experiments show that the snRNAs may be quantitatively precipitated from nuclear extracts in the

form of these ribonucleoprotein complexes. The authors propose that each snRNA can interact with the same set of seven polypeptides to form a discrete ribonucleoprotein particle (snRNP).

A further step in the characterisation of the snRNPs is described in this issue of *Nature* (Lerner *et al.*). First it is shown that the lupus antisera precipitate similar snRNP complexes from a variety of eukaryotic sources, ranging from man to insect, indicating that these complexes have been highly conserved during evolution. They are not found, however, in tobacco, yeast, *Dictyostelium discoideum*, or *E. coli* extracts. They are present in highest abundance in metabolically active cells, and from such cells they sediment, in sucrose gradients, with a molecular weight (30S) far greater than that observed (7-10S) for the free snRNP species. This suggests that the 7-10S particles are themselves bound to some other moiety of much higher molecular weight. The authors speculate that the high apparent molecular weight may be due to the attachment, of the snRNP particles, to hnRNA, which is itself associated with 30S hnRNP particles.

The arguments leading to this prediction, while not compelling, are highly intriguing. Several groups have examined the sequences present around the splice junctions in hnRNA and defined a consensus sequence from which any one sequence diverges by only a few nucleotides. This is postulated to be a 'preferred' sequence at which splicing could occur. By examining the sequence of a major snRNA (U1A), the authors noted that its 5'-terminal sequence shows extensive complementarity to this preferred sequence. Nucleotides 3 to 8 from U1A exactly match that part of the 'preferred' sequence found at the 5' end of the intervening sequence, while nucleotides 9 to 11 exactly match the 3' end of the intervening sequence. Some further possibilities for base pairing exist between nucleotides 12 to 20 of U1A RNA and the

continuation of the 3' intervening sequence. Thus it is hypothesised that the 5'-terminal sequences of U1A RNA interact with the terminal sequences of the intervening regions and thus serve a template function by correctly aligning the sequences to be spliced. Consistent with this role for the 5'-terminal sequences of U1A RNA is the finding that among the snRNP particles is a minority class which does not show the high apparent molecular weight in sucrose density gradients. This class contains a shortened form of U1A RNA which is missing both the 5' terminal 'cap' structure and the first six nucleotides from the 5' end. The absence of these nucleotides would seriously disrupt the fit required by the model. It is not known whether these particles, containing the shortened form of U1A RNA, are an artefact of isolation or have metabolic significance.

A similar scheme has previously been proposed in the adenovirus-2 (Ad2) system, this time invoking the Ad2 encoded VA RNA as a template for the processing of late Ad2 mRNAs (L. Philipson, unpublished *News & Views* **277**, 593; 1979; Murray & Holliday *FEBS Lett.* **106**, 5; 1979). Here, the detailed model of Murray and Holliday seems less attractive since it requires base pairing with nucleotides which lie beyond the known 3' end of VA RNA. Whatever the final outcome of these predictions, it seems that at last an experimental system is available for the study of RNA processing events within the eukaryotic nucleus and such studies should be spurred by the tantalising hypothesis developed in this latest report.

Amidst the frenzy of activity presently aimed at cloning and sequencing DNA, we should perhaps remember that many of the highly conserved processes of molecular biology involve only RNA and protein. The specificity of protein synthesis is provided by the triplet interaction between tRNA and mRNA, while its initiation requires RNA-RNA interaction between the 3' end of ribosomal RNA and mRNA. The ribosome which catalyses this process contains both RNA and protein and recently an RNA processing enzyme,

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RNase P of *E. coli*, has also been shown to consist of both RNA and protein (Stark *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3717; 1978). Some of us would argue that this reflects the early establishment of these processes during evolution, at a time preceding the arrival of DNA on the

biological scene. If RNA splicing is also a process which developed at an early stage in evolution (*News & Views* **272**, 581; 1978; Darnell, *Science* **202**, 1257; 1978) then it may not prove surprising if its specificity also depends upon RNA-RNA interactions. □

## Galaxies: the origin of spiral structure

from M.G. Edmunds

A RECENT comparison of the morphology and rotational properties of nearby galaxies, together with computer simulations of a simple model of patterns of star formation, may have greatly clarified our understanding of the causes of the beautiful spiral structures seen in many large systems. The origin of such structure remained a source of speculation for many years until during the 1960s the formulation of a 'density wave' theory seemed to provide a suitable physical mechanism. A clue lay in the fact that well-developed spiral structure is seen only in galaxies with disks containing both stars and gas. The basis of the theory is the propagation around the centre of the galaxy of a perturbation in the space density of stars. The gas in the interstellar medium responds strongly to the perturbation in the gravitational field, and its compression results in a shock wave which instigates the gravitational collapse of gas and dust to form stars. It is the very non-linear behaviour of the gas which gives rise to the large influence on the overall appearance of a galaxy for only a small perturbation in the underlying distribution of stars. Of the stars which form, the massive ones are extremely luminous, and it is they which are seen delineating the spiral pattern. Such stars are short lived and soon die out, leaving the wave to show itself by new star formation as it moves relative to the interstellar gas. The pattern of the wave rotates 'rigidly' around the galaxy's centre, although the gas in stars may be in differential rotation — and hence relative shearing motion. The extent of the differential rotation is governed by the overall gravitational field experienced by the stars and gas particles.

Unfortunately a fundamental difficulty arose in understanding how the spiral wave manages to maintain itself against dissipation. A particularly important dissipating process will occur at the so-called 'inner Lindblad resonance'. A star in a galaxy will have a bounded orbit around the centre, but the orbit will not normally be either circular or closed. The intervals between the star being at some particular distance from the centre will, however, be

well defined with the frequency of successive inward or outward passages being called the 'epicyclic' frequency, and this is determined by the local gravitational field. Now if the frequency of successive passages past the star of the density wave perturbation in gravitational field exactly matches the epicyclic frequency at that radius, then the star will experience acceleration due to the perturbation at the same point on successive orbits. A resonance will hence occur which will feed

energy out of the density wave and into the velocities of the resonated stars. The problem for the density-wave theory is to replace this loss of energy, and no obvious method exists for a single isolated galaxy with a simple pure spiral pattern. A way round the difficulty would be to show that isolated spiral galaxies which show good spiral structure do not have inner Lindblad resonances. No resonance would occur if there were no differential rotation, since the pattern speed in this case will never match the epicyclic frequency.

Such behaviour has indeed been found by J. Kormendy and C.A. Norman (*Astrophys. J.* **233**, 539; 1979) in a discussion of the morphology of some 54 spiral galaxies for which rotation velocities are available. Those galaxies which are on their own and have well-developed spiral patterns do not differentially rotate over the region where the spiral structure is apparent. The galaxies must be able to find enough energy transfer to overcome any other (less important) dissipation of the wave. But these galaxies represent only one-fifth of the sample. Over half the sample do differentially rotate and hence are expected to have an inner Lindblad resonance. Yet they still show good spiral structure, the difference being that the differential rotators either have a nearby companion galaxy or else have a bar structure in their centres. An example of a barred galaxy is shown in Fig. 1, and a less

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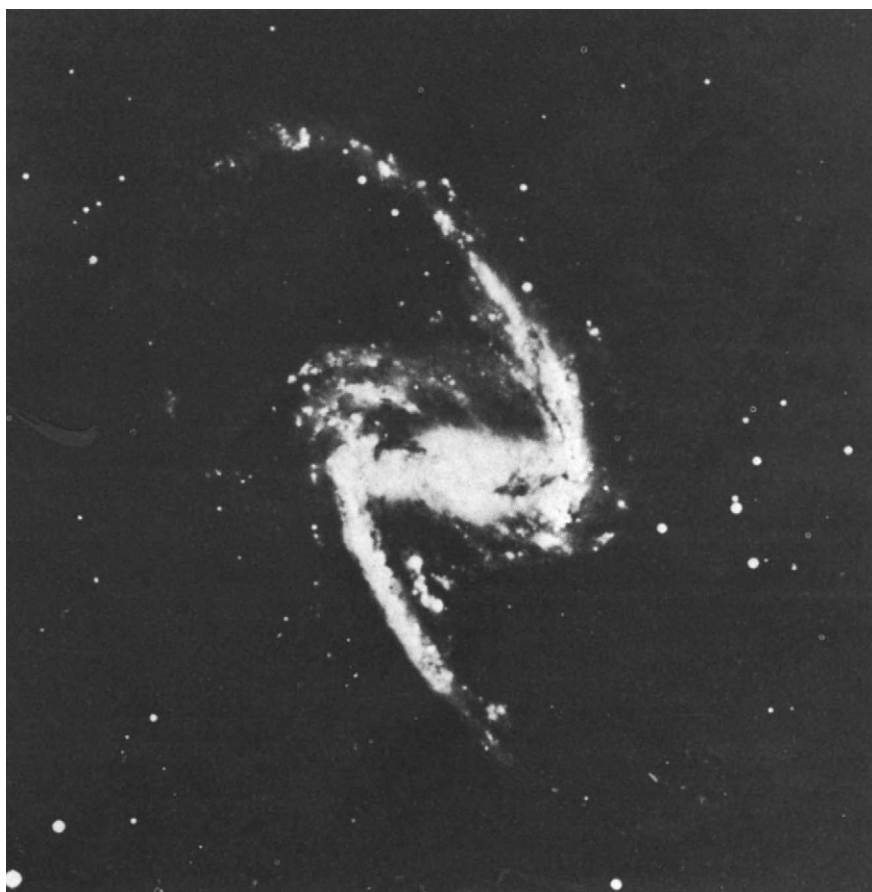


Fig. 1. NGC 1365 showing a well-developed bar and two-armed spiral structure.

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extreme (but still barred) case in Fig. 2. The importance of the companion or bar is in providing a significant rotating non-axisymmetric gravitational potential which will 'stir' the galaxy and force the spiral structure. The pattern is still the result of a density wave, but the driving of the wave will presumably replenish the energy lost at the inner Lindblad resonance. The initial formation of the bar presents no problems, since numerical simulations of the stellar dynamics in galaxies show a particular willingness to evolve rapidly to give a central bar.

There still remains a significant fraction of galaxies in the sample which differentially rotate, but have neither bar nor companion. An example of the morphology of this type of galaxy (although not one from Kormendy and Norman's sample) is shown in Fig. 3. The spiral structure is very fragmentary, with no grand two-armed design, but rather a system of multiple and branched arms which can only be individually followed over short distances. P.E. Seiden and H. Gerola (*Astrophys. J.* **233**, 56; 1979) have developed a series of computer models which nicely simulate such structures, and show that it may have very little at all to do with a global density wave pattern. Their model supposes that the supernova explosion of the bright massive stars which illuminate spiral structure can produce further star formation, since the shock which the explosion pushes out into the interstellar medium may induce the collapse of nearby interstellar clouds. The new massive stars formed would subsequently also explode, and so the star formation would propagate through the galaxy. Strong differential rotation of the disk would tend to pull such regions of star formation out into an arc — causing a short



Fig. 2. NGC 5236 (M 83) showing a less obvious, but still important, central bar.

spiral arm. An element of randomness is introduced by allowing some spontaneous star formation, and the correlated activity which gives rise to structure is produced by having a higher probability for the supernova-induced star formation. Since there is some evidence from supernova

remnants in our own Galaxy that such induced star formation can occur, Seiden and Gerola's model is attractive, and must also play some part in influencing the morphology of spiral arms even when a large-scale density wave is operative.

Life would not be so interesting without exceptions, and two unusual galaxies turn up in Kormendy and Norman's sample. These are both isolated single-arm (as opposed to normal double-arm) and they differentially rotate, although the spiral arm in one of them (NGC 4378) can be traced over almost  $1\frac{1}{2}$  revolutions. But the fact that only two cases out of a sample of 54 cannot be immediately classified is surely encouraging.

There remain lingering theoretical problems connected with preventing a stellar disk from becoming too unstable to bar formation without either an unacceptably large velocity dispersion among the stars or a rather *ad hoc* massive halo of low luminosity objects surrounding the disk. Despite this, the three mechanisms — a self-sustaining two-armed density wave in the absence of an inner Lindblad resonance, a density wave driven by the gravitational perturbation from a stellar bar or companion galaxy, or stochastic supernova-induced star formation with differential shear in the disk — seem to provide a reasonable basis on which to account for the structure of spiral galaxies. □



Fig. 3. NGC 7793 showing multi-armed, filamentary spiral structure.



# Carcinogens and DNA

from Stephen Neidle

THE physical and chemical properties of the potent diolepoxide carcinogens have been much studied in the hope of determining the molecular basis of their carcinogenic and mutagenic effects. They are known to bind directly to DNA, and this reaction has formed the focus of both experimental and theoretical investigations into the activity of diolepoxides such as benzo(a)pyrene 7,8 dihydrodiol 9,10 oxide, the most important active carcinogenic metabolite of benzo(a)pyrene.

Although theoretical approaches to the likely conformation and chemical reactivity of the four possible stereoisomers of benzo(a)pyrene diolepoxide, using quantum-mechanical methodology, have had great influence on thinking in this field, in many cases the theoretical findings conflict with the experimental evidence and it is fair to say that only some of the recent theoretical treatments are successful or other than trivial in their predictions. The deficiencies and limitations of some approaches are highlighted for example, by their inability to explain satisfactorily the considerably greater tumorigenicity and DNA binding ability of the *anti* compared with the *syn* isomer (Brookes *Cancer Lett.* 6, 285; 1979), even though the theoretically-derived data indicate that the *syn* isomer is the more stable (Klopman, Grinberg & Hopfinger *J. theor. Biol.* 79, 355; 1979) and until relatively recently was believed to be the biologically significant one.

Application of the  $\phi$ X174 DNA assay system developed by Harvey and coworkers to a series of diolepoxide carcinogens, has led to the revelation of further deficiencies in some quantum mechanical analyses (Hsu *et al. Biochem. biophys. Res. Commun.* 87, 416; 88, 1; 1979). This assay system is remarkably effective in correlating inhibition of viral infectivity with carcinogenic potency of the various diolepoxides, yet this correlation was not followed either by predictions of reactivities from molecular orbital theory or by the relative structure positions of the diolepoxide groupings on the hydrocarbon skeletons.

Clearly, studies of the chemicals themselves are not enough to explain their different carcinogenic potencies and other factors such as the geometry and kinetics of their binding to the DNA receptor sites will have to be taken into account.

Benzo(a)pyrene diolepoxide carcinogenesis is generally considered to involve direct binding to DNA, with guanine residues as the major sites of attachment at

least in terms of stoichiometry. About 10% of the total binding is to adenine however, and although its significance is unclear, some recent evidence correlates binding to adenines and not to guanines with mouse skin tumorigenicity (Di Giovanni *et al. Cancer Lett.* 7, 39; 1979), although in this report no adducts have actually been characterised as diolepoxide ones. Adenine-modified regions of DNA are known to have rather different conformational properties in terms of greater local denaturation than their guanine counterparts. The *anti* form of the diol epoxide can be resolved to two stereoisomers. Their interactions with DNA are, not surprisingly, stereospecific (Meehan & Straub *Nature* 277, 410; 1979), with essentially only the (+) isomer binding to double-stranded DNA (to guanines) whereas denatured, single-stranded DNA was equally susceptible to both isomers. These differences in binding ability correlate with biological activity in several cell lines — the (+) isomer being more carcinogenic — and Meehan and Straub suggest that DNA secondary structure is an important determinant in carcinogenicity.

One question of interest in looking at the binding of carcinogens to DNA is whether any specific base sequence is involved. Weinstein, Grunberger and coworkers (*Biochemistry*, in the press) do to a large extent confirm Meehan & Straub's findings, with the additional important observation from restriction enzyme analysis of modified SV40 and lambda DNA, that no appreciable specificity of binding in respect of base sequence was apparent. However, subtle and possibly important effects of binding specificity may occur. Probably more crucial to carcinogenicity is the geometry of the binding in relation to subsequent excision repair by endonucleases. Binding to different residues and different chemical groups within a residue dramatically affects excisability. Increased excisability implies attachment on the outside rather than to deeply buried sites within the molecule.

There is no lack of molecular models for the structure of benzo(a)pyrene diol epoxide-modified DNA, some of which owe more to imagination than to reality. The recent suggestions of Beland (*Chem.-Biol. Interactions* 22, 329; 1978) are among the more plausible and detailed with the benzo(a)pyrene chromophore being situated in the DNA minor groove and causing some local denaturation of the double helix. This general type of non-intercalative model is strongly supported by several recent physical measurements on the diol epoxide-DNA complex. Fluorescence probe techniques (Prusik *et al. Photochem. Photobiol.* 29, 223; 1979; Prusik & Geacintov *Biochem. biophys. Res. Commun.* 88, 782; 1979) give data consistent with the benzo(a)pyrene chromophore attached (by N2 of guanine) externally to the DNA double helix, and

oriented with at most a 35° angle to the helix axis. In particular, intercalation (at least in the sense of the classical Lerman model) is ruled out by these workers on account of the accessibility of the chromophore to external probedings. Additional evidence for a non-intercalative model is also provided by the new technique of optically-detected magnetic resonance (Lefkowitz *et al. FEBS Lett.* 105, 77; 1979).

Unfortunately such techniques cannot provide detailed information on the molecular geometry of the modified residues of DNA. The situation is made yet more complex by the growing realisation that DNA structure is not always necessarily of the classical forms. There is now powerful evidence for example, that tracts of alternating pyrimidine-purine residues have highly unusual structures (see for example *Nature* 282, 680; 1979; *News and Views*, 283, 11; 1980); it would not be surprising if binding of carcinogens did not sometimes exploit such peculiarities. Molecular model-building of nucleic acid-carcinogen interaction urgently requires input of firm data from the fine structure techniques of X-ray crystallography and NMR in order to achieve credibility. Questions of the relevance of the structural peculiarities mentioned above to differences in excisability and repair, and to carcinogenic and mutagenic processes in general must await future investigations. □

## The specificity of plant defences

from R.A. Dixon & C.J. Lamb

WHEN plants are attacked by bacterial or fungal pathogens they synthesise and accumulate nonspecific antimicrobial compounds known as phytoalexins. A few years ago it was established that phytoalexins could be induced to accumulate by glucan and glycoprotein 'elicitor' molecules associated with the cell wall of the invading pathogen (see *News & Views* 273, 266; 1978) and this has started a search for the molecular basis of the exquisite specificity seen in the classical 'gene-for-gene' relationship between plant cultivars and physiological races of a pathogen, looked at in terms of elicitors and phytoalexin production.

In the simplest terms the gene-for-gene relationship consists of a gene for 'avirulence' in the pathogen being complemented by a specific one for resistance in the plant — the plant and pathogen are in this case incompatible.

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Disease results when a mutation in the pathogen produces a new avirulence gene (now a 'virulence' gene) that is not matched by the available resistance genes carried by the host cultivar — the plant and pathogen are now compatible. Plant breeders have long been familiar with the ability of plant pathogens to keep one jump ahead by continually throwing up new races to which hitherto resistant varieties of crops are completely susceptible.

The simplest model for the gene-for-gene phenomenon in terms of elicitors requires that a race-specific elicitor (presumably the product of the pathogen's avirulence gene) is recognised by a specific receptor on the plant cell (presumed to be the product of the plant's resistance gene); phytoalexin production is induced and the pathogen is limited. If a mutation either in the pathogen or plant means that the elicitor is no longer recognised by the plant, then the infection is not limited and disease follows. The key predictions of this model are first, the existence of race-specific elicitors, and second, that the differential accumulation of phytoalexin is the most important determinant of disease resistance.

Recent work suggests that this model is indeed an oversimplification and that the real world is considerably more complex. On the question of race-specific elicitors Keen *et al.* (*Physiol. Plant Pathol.* **15**, 43; 1979) recently claimed to show race-specific elicitors in the cell envelopes of physiological races of the bacterium *Pseudomonas glycinea*, which causes bacterial blight in soybeans. High molecular weight glycoproteins solubilised by detergent treatment showed the same specificity, with one exception, in eliciting accumulation of the phytoalexin glyceollin in cotyledons of two varieties of soybean as did the bacterial races from which the elicitors were isolated.

In contrast, however, Albersheim and coworkers (*Plant Physiol.* **57**, 760; 1976) have shown that glucan elicitors from four different races of the fungal pathogen *Phytophthora megasperma* var. *sojae* (Pms) are qualitatively and quantitatively identical suggesting that further 'specificity factors' are needed to account for race-specific resistance. A recent report by Wade and Albersheim (*Proc. natn. Acad. Sci. U.S.A.* **76**, 4433; 1979) now identifies glycopeptides secreted into the culture medium by Pms as the proposed factors. Glycopeptides from three races of Pms were tested for their ability to prevent the expression of serious symptoms in four soybean cultivars. Glycopeptides from incompatible races of the pathogen but not from compatible ones protected seedlings from attack by compatible races. Glycopeptides from compatible races did not cause cultivars resistant to a given Pms race to become susceptible to that race. The glycopeptides themselves are only weak elicitors and their effect on glucan elicitor action remains to be established. Wade and

Albersheim postulate that synthesis of the glycopeptides is controlled by the pathogen's avirulence genes and that in incompatible but not compatible interactions they trigger a defence reaction which decreases the rate of pathogen development and enables inhibitory levels of phytoalexin to accumulate in the area of initial fungal penetration.

Such a hypothesis still leaves open the nature of the proposed defence reaction and the product of the host's resistance gene. This model assumes no determinative role for elicitor-induced phytoalexin accumulation in the soybean-Pms system and indeed Albersheim and coworkers (*Plant Physiol.* **57**, 760; 1976) have shown that initial rates of glyceollin accumulation are identical during infection of wounded hypocotyls by compatible and incompatible races of Pms. This contradicts earlier work of Keen (*Physiol. Plant Pathol.* **1**, 165; 1971) who observed differential rates of glyceollin accumulation in this system. However, in both studies no account is taken of the cellular and subcellular distribution of glyceollin and such considerations may be crucial for a full understanding of the role of phytoalexins in race-specific resistance.

A common feature of the models of Keen and Albersheim is that specificity is associated with the incompatible interaction. However, this may not always be the case. Two recent papers by Kuć and coworkers (*Physiol. Plant Pathol.* **15**, 117 and 127; 1979) suggest that in potato cultivars with R genes, specificity in resistance to physiological races of *Phytophthora infestans*, the causal agent of potato blight, is associated with compatibility, and a phytoalexin suppressor, or 'hypersensitivity inhibiting factor' is proposed as the determinant of specificity. They found that mycelia and zoospores of a compatible race of *P. infestans* contain  $\beta$ , 1-3,  $\beta$ , 1-6-linked low molecular weight glucans which could inhibit the rapid cell death, loss of electrolytes, tissue browning and accumulation of terpenoid phytoalexins associated with the hypersensitive reaction of potato tissues to treatment either with elicitor from the same compatible race or infection by an incompatible race. Although the incompatible race contained similar glucans they were much less active as suppressors of the hypersensitive reaction.

The glucans consisted of non-anionic and anionic fractions, the latter containing a small proportion of glucose residues as phosphoryl monoesters. In preliminary experiments, mixing elicitors from compatible or incompatible races with a membrane preparation from potato tubers led to decreased elicitor activity on subsequent application to tuber tissues, possibly as a result of sequestration of elicitors by binding sites on the membrane preparation. Only glucans from the compatible race were able to restore elicitor

activity, suggesting that the small glucans may compete for the elicitor binding sites. These molecules may prove valuable tools in the search for elicitor receptor sites. Elicitors from *P. infestans* agglutinate potato protoplasts indicating cell surface binding sites (*Science* **201**, 364; 1978), but it is not clear whether these sites are receptors for elicitor induction of phytoalexin accumulation.

Although no unified hypothesis has yet emerged, the demonstration of molecules that determine specificity in plant-pathogen interactions is a major development in the study of the molecular basis of race-specific disease resistance. Clearly there is considerable scope for collaboration between biochemists and pathologists in this important field. □

## Patterns of ungulate reproduction

from Robert M. May

ONE theme, around which much current ecological research is organised, concerns the relation between an animal's physical and biological environment (its habitat and the species it interacts with) and its foraging and reproductive behaviour. In this context, Robbins and Robbins (*Am. Nat.* **114**, 101; 1979) have recently drawn together a lot of data on ungulate and subungulate species, with a view to elucidating fetal and neonatal growth patterns, and trends in maternal reproductive effort. These authors believe ungulates to be especially interesting, because they inhabit a wide range of environments, and comprise species in many different stages of domestication.

By plotting the weight of the fetus (expressed as a fraction of the weight at parturition) as a function of time (expressed as a fraction of the gestation period) for several species, Robbins and Robbins show the fetal growth rate in ungulates is a power function that varies little among species, and is typically of the general mammalian pattern derived by Sacher and Staffeldt (*Am. Nat.* **108**, 593; 1974) and others. Using data for white-tailed deer, domestic sheep and cattle, they also show the weight of the actual fetus, as a percentage of the gravid uterus, increases steadily throughout gestation, to around 60% at parturition. The pattern shows no significant differences between natural and domesticated ungulates; that is, selection for a larger neonate in domesticated animals entails a proportional enlargement of the uterus, fluids and membranes. As Robbins and Robbins point out, this fact

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suggests "The efficiency of the reproductive process in terms of fetal energy or matter produced per unit of uterine tissue may already be selectively maximized" in natural populations. Similarly, under maternal nursing the maximum growth rates of newly born animals ('neonates') of different species increase in a systematic fashion with increasing maternal weight, with no significant differences between natural and domesticated ungulates. Estimates of the average milk production at the peak of lactation by well-nourished females, nursing a single neonate, also reflect no more than a systematic, curvilinear increase with increasing maternal weight.

The relationship between weight of offspring at birth (expressed as a percentage of maternal weight, or 'proportionate birth weight') and average maternal weight is shown in Fig. 1, for some 69 species. We see the maximum relative reproductive effort apparently occurs in pronghorn antelope and springbok, at around 15%, and decreases to around 4-5% for the very large ungulates and subungulates. The trend to diminishing proportionate birth weight with increasing maternal weight is fairly steady for the larger animals, but there is much variability among the small species (adult weights less than or around 100 kg). Although there is too much scatter for any crisp generalisation, Fig. 1 shows that several north temperate and arctic

ungulates (for example, caribou, muskox, bighorn sheep) tend to produce relatively smaller offspring than most African ungulates, with North American species typically being intermediate. The explanation for the relatively small single fetus of caribou, muskox and bighorn sheep may lie in "Inadequate food supplies during winter and the need to optimize maternal survival relative to reproductive effort"; these factors apparently override thermodynamic considerations, which would argue for the largest possible neonate, to minimise heat loss in a cold environment.

As Robbins and Robbins emphasise, there are yet further complications among the smaller species represented in Fig. 1. The Mexican red brocket deer, a shy tropical animal which has one of the lowest proportionate birth weights of the smaller ruminants, has a postpartum estrus. So do muntjac deer and several African bovids. These species live in relatively stable environments, and the combination of relatively low birth weight and postpartum estrus enables them to distribute reproduction throughout the year, in a way that may well increase total reproductive output.

In short, Robbins and Robbins' comparisons among species of ungulates and subungulates suggest that "very basic controlling mechanisms" may produce systematic patterns in fetal and neonatal growth, with these patterns being common

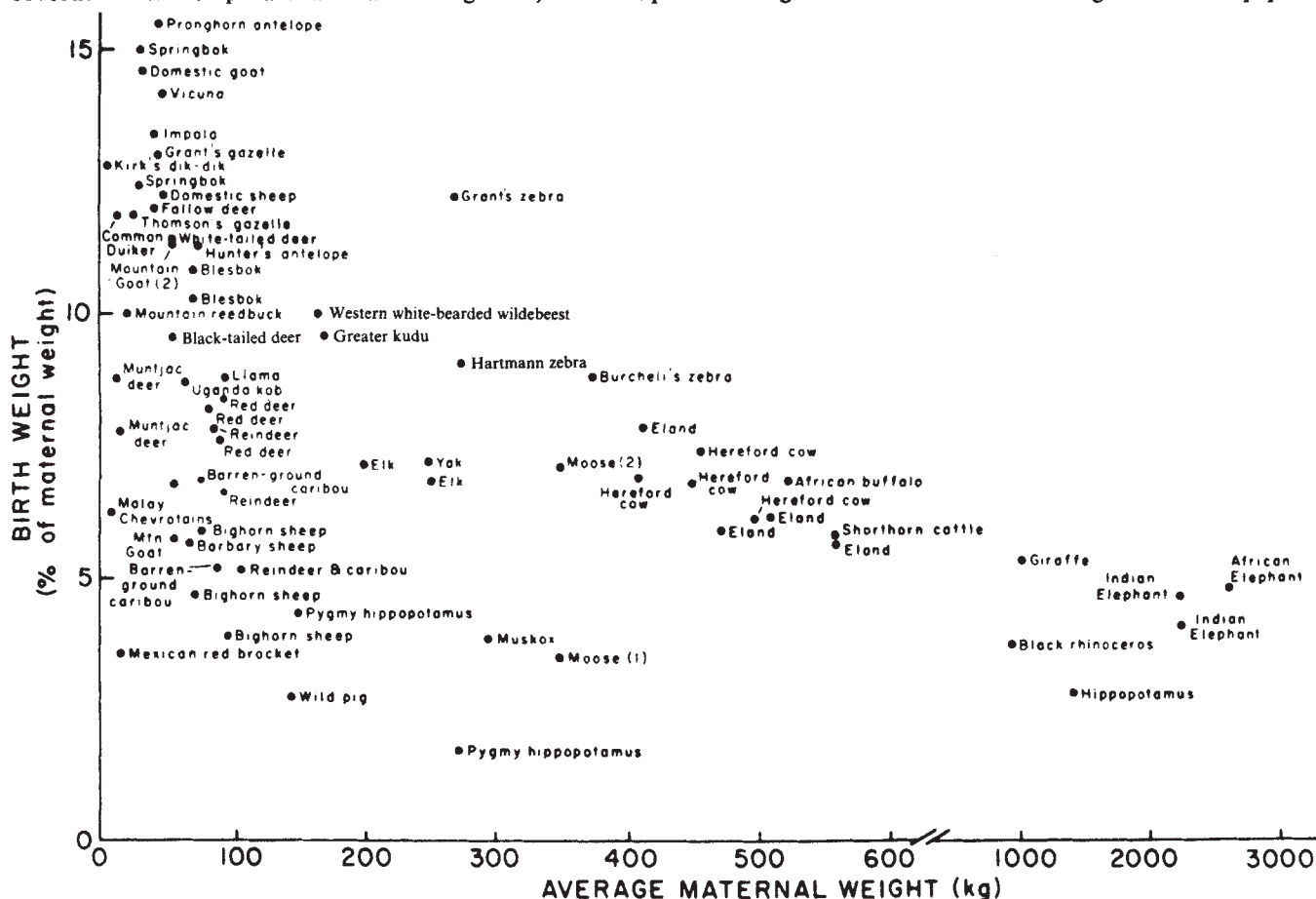
to natural and domesticated species. The proportionate birth weights of the smaller species, however, tend to exhibit a fascinating richness of detail that overwhelms any easy generalisation. □

## The non-linear spiral

*from J. David Pye*

**THE** sense organ of the ear is the cochlea which, as its name implies, is shaped like a snail's shell. Inside its hard, bony wall are three liquid-filled chambers that run together round the spiral from the base to the tip. One of the dividing partitions is a very thin, acoustically transparent membrane but the other, the basilar membrane, is an elaborate structure. It bears four rows of sensory hair cells that are connected by synapses to the ends of nerve fibres that run to the brain.

Sound-induced vibrations of the eardrum are efficiently transmitted to the incompressible cochlear liquids by a chain of three tiny bones. A relatively slow travelling wave is then initiated in the basilar membrane and its movement excites the hair cells. For high-pitched tones the travelling wave builds up quickly



**Fig. 1** Birth weight (as a fraction of maternal weight) as a function of average maternal weight in ungulate and subungulate species.

to a peak near the base of the cochlea and then decays while for deeper tones it runs further towards the tip, thus exciting a different population of hair cells.

The cochlea therefore, not only transduces sound energy into nerve signals to the brain but also acts as a mechanical frequency analyser. But despite extensive study, neither of these functions is well understood in detail, for the known properties have so far not yielded a satisfactory synthesis. New findings from several sources now suggest that some parts of the mechanical action are non-linear and this realisation has greatly stimulated both theoretical speculation and new kinds of observations.

The new developments were aired at a recent meeting in London\* and some of the highlights are mentioned below. Non-linearity implies that a response is not directly proportional to its cause (the response curve is not straight); it also leads to the local generation of distortion products that are not present in the original stimulus. This is of course a common feature of any system that is 'overdriven' by excessive stimuli and indeed the beat-notes and other combination tones produced in the ear by loud sounds are already familiar. It is surprising, however, to find that the ear is markedly non-linear even at moderate and low sound intensities and that this property is related to active processes in the living organ.

Conventional linear models of cochlear mechanics have always presented problems and non-linear models which embrace some previously unexplained phenomena have been proposed. Indirect evidence of non-linear motion has been obtained by electrophysiological and acoustic techniques (D. O. Kim, Washington University).

Direct evidence of non-linearity has been provided by E. Lepage and B. Johnstone of the University of Western Australia, Perth, who measured movements of the cochlear partition in guinea pigs by a micro-capacitative probe. They were able to confirm the results of W.S. Rhode of the University of Wisconsin, Madison who had previously demonstrated non-linearity in monkeys by a less sensitive Mössbauer technique.

Further direct evidence was provided by D.T. Kemp (The Royal National Hospital, London) who discussed in detail his newly discovered mechanical evoked response or 'cochlear echo'. He and his collaborators have shown that brief sounds introduced into the ear canal can be detected again several milliseconds later as a vibration of the eardrum coming out from the cochlea. This is thought to occur because active non-linearity in the mechanical action of the basilar membrane would produce local changes in its impedance from which some

of the energy will be reflected back towards the base of the cochlea. The involvement of active mechanical processes, using energy derived from physiological sources, was unexpected; but measurements of the echo show that under certain conditions it may contain more energy than is available from a passive reflection from that point. The theories of passive non-linearity that originally predicted the effect have therefore had to be revised.

The phenomenon has been independently confirmed by other workers (J.P. Wilson, University of Keele; H.P. Wit & R.J. Ritsma, University Hospital; Groningen; W.L. Rutten, Academic Hospital, Leiden; Kim). As the cochlear 'Kemp echo' has been shown to depend on normal physiological functioning of the living cochlea, its measurement can form a valuable new diagnostic tool. It is also related to the fine structure of the normal hearing curve (which is not smooth but shows regular, very sharply tuned peaks of up to 10 dB) and both responses can be detected by instruments at sound levels far below the audible threshold (Wilson). That the echo originates from mechanical non-linearity is further demonstrated by its relationship to the distortion products, especially the difference tone ( $f_2 - f_1$ ) and the cubic term ( $2f_1 - f_2$ ). Finally, it seems to be intimately linked with certain forms of tinnitus, where the cochlea oscillates spontaneously (for zero stimulus energy) and produces an objective tone that can be recorded from the eardrum.

In the field of psychoacoustic and electrophysiological observations, particular attention is being devoted to the physiologically vulnerable combination tones ( $f_2 - f_1$  and  $2f_1 - f_2$ ) and to the elusive 'second filter' that makes the responses of single nerve fibres more sharply tuned than the basilar membrane that excites them. Here again the properties of the cochlear echo (in response to paired stimulations)

may provide a valuable new kind of evidence. But A.R. Palmer and E.F. Evans (University of Keele) found that single unit recordings from the acoustic nerve gave no evidence of mechanical non-linearity in the cochlea.

In discussions of cochlear mechanisms, of especial interest was the demonstration by A. Flock (Karolinska Institute, Stockholm) that the simple stereocilia of the hair cells are filled with actin filaments which also occur in the cuticular plate and round the apical circumference of the cell itself. The discovery of contractile proteins at these sites could be of great significance in understanding the transduction from mechanical to nervous (that is, electrical) energy and the part played by active, non-linear mechanical processes.

A.C. Crawford and R. Fettiplace (Cambridge University) showed that electrical stimulation of hair cells in the terrapin produces a finely tuned electrical response similar to that elicited by sound. The very short cochlea in the terrapin is probably an ineffective mechanical analyser and this discovery suggests that the second filter involves cellular processes. In the more traditional guinea pig, I.J. Russell and P.M. Sellick (University of Sussex) and Y. Tanaka (Dokkyo University, Tichigi) and his colleagues showed that the inner and outer rows of hair cells have very different response properties that may contribute separately to the final output of the cochlea.

It seems safe to predict that this meeting will come to be regarded as a milestone in the study of hearing. Hi-fi enthusiasts may be shocked, but non-linearity of the normal, healthy cochlea could be a key to its amazing performance. □

\*A meeting on non-linear and active mechanical processes in the cochlea was held on 17-19 September, 1979 at the Institute of Laryngology and Otology of the Royal National Throat Nose and Ear Hospital, London. It was organised by Dr D.T. Kemp and Mr S.D. Anderson.



### 100 years ago

We pass on therefore to the lower marine animals, and select as an example of the way they are treated a rare British species allied to the sea cucumbers, and named *Synapta duvernea*. It was discovered in the English Channel by M. Quatre-fages, who thus describes it:-

"Imagine a cylinder of rose-coloured crystal as much as eighteen inches long and more than an inch in diameter, traversed in all its length by five narrow ribbands of white silk, and its head surmounted by a living flower whose twelve tentacles of purest white fall behind in a graceful curve.

From *Nature* 21, 8 Jan., 234; 1880.



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## REVIEW ARTICLE

## The pathogenesis of cancer metastasis

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*Metastases do not result from random survival of cells released from the primary tumour but from the selective growth of specialised subpopulations of highly metastatic cells endowed with specific properties that befit them to complete each step of the metastatic process.*

UNDERSTANDING how malignant tumour cells are able to spread from the primary tumour to form secondary tumours (metastases) at other sites in the body is one of the major goals of cancer research. Despite advances in surgical technique and the development of aggressive adjuvant therapies for treatment of primary neoplasms, most deaths in cancer patients are caused by metastases. Research done in many laboratories in the last few years has provided important new information about basic events in the pathogenesis of metastasis and the identity of the tumour cells responsible for this fatal disease. A number of these observations challenge long held views and raise serious doubts about the adequacy of certain experimental approaches used to date to study the malignant cell. This article reviews some of these findings and their implications for the experimental analysis of the malignant phenotype and the development of agents for treatment of metastatic disease.

## The metastatic process

A metastasis is a neoplastic lesion that arises from another cancer with which it is no longer in contiguity. The ability to produce metastases is a property of malignant tumour cells. Malignancy is not a characteristic shared by all tumour cells and cells that lack the ability to invade and metastasise are termed benign. This classification may seem too obvious for inclusion in a review article on metastasis. However, the most cursory examination of the literature reveals that the term 'malignant' is being used increasingly, albeit incorrectly, as a synonym for 'neoplastic' or 'tumourigenic'. It is also common to see publications that erroneously refer to 'malignant transformation' of cells *in vitro*. In far too many instances this description is based on inadequate criteria such as changes in cell growth *in vitro* or biochemical alterations and experiments to determine whether such cells can cause tumours *in vivo* (that is, neoplastic transformation) yet alone produce metastases (that is, malignant transformation) have not been done. In this article the term 'malignant' refers only to cells that are capable of producing metastases.

The process of metastasis involves a series of sequential steps<sup>1-3</sup> in which malignant cells are released from the primary tumour and disseminate to distant sites where they proliferate to form new tumour foci (Fig. 1). Metastasis begins with the local invasion of the surrounding host tissue by cells from the primary tumour, either singly, or as cell clumps, or as large multicellular tongues<sup>1</sup>. It is not known exactly how malignant cells invade host tissues. Active and/or passive movements of tumour cells into host tissues are probably important<sup>1</sup>. The ability of some malignant cells to release tissue-destructive enzymes, particularly lysosomal hydrolases and collagenolytic enzymes, has also attracted attention as a possible factor in promoting tumour invasion<sup>6-12</sup>. However, as with virtually all aspects of tumour biology, generalisation is difficult and malignant cell lines exhibit a broad range of tissue digestive capacities<sup>6-10</sup>.

In certain neoplasms, distant tumour foci are established by a direct spread of cells from the primary lesion along preformed mechanical pathways such as fascia planes and nerve sheaths<sup>2,12</sup>. Similarly, for tumours growing in major body cavities, shedding of cells from the primary lesion can produce secondary growths by seeding of cells onto the mucosal and/or serosal surfaces of other organs<sup>12</sup>.

The more common, and most important, route of tumour spread involves invasion and penetration of tumour cells into blood vessels and/or lymphatics, with their subsequent dissemination to distant organs in the blood or lymph. Lymphatics and small, thin-walled venules offer relatively little mechanical resistance to penetration by tumour cells<sup>2-5</sup>. In contrast, arteries and arterioles are rarely invaded<sup>5</sup>. Blood vessels within tumours also offer malignant cells a port of entry into the circulation. Ultrastructural studies indicate that the endothelium of tumour-associated vessels is often defective and such vessels may be particularly susceptible to penetration by tumour cells<sup>13</sup>. Zones of reduced oxygenation<sup>14</sup> and overt necrosis<sup>3,9,12</sup> are common in tumours and the blood vessels in such regions show structural alterations (defects) that could facilitate invasion by tumour cells.

Some of the clinical literature still pays homage to the view that carcinomas tend to spread via lymphatics, whereas malignant tumours of mesenchymal origin spread via the blood system. There is now substantial evidence that malignant cells can pass freely between lymphatics and blood vessels<sup>2-5</sup>. The belief that particular tumours spread exclusively via one or other pathways is a misleading oversimplification, which does not offer a reliable basis for predicting either the distribution of metastases or the choice of therapy<sup>4</sup>.

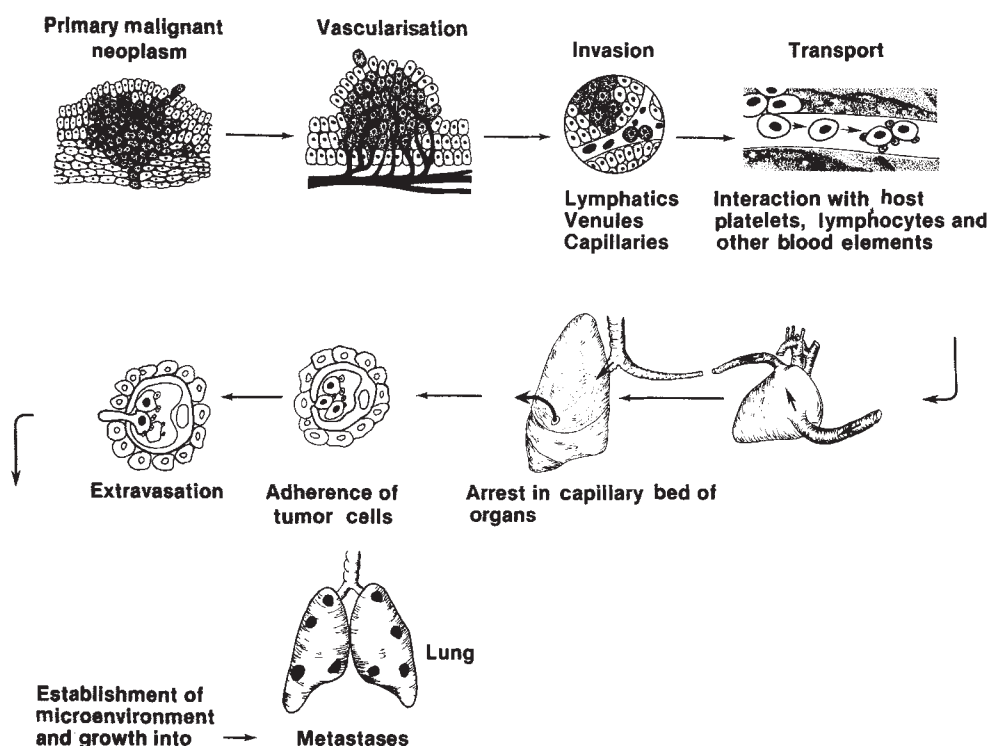
Following penetration of blood vessels, tumour cells are either carried away passively in the bloodstream or remain at the site of vessel invasion where they proliferate and continue to shed emboli into the circulation<sup>2</sup>. Shedding of emboli is exacerbated by intermittent changes in venous pressure, by turbulent alterations in blood flow and by movements or manipulation of the neoplasm during diagnostic tests or surgery<sup>15</sup>.

## The distribution of metastases

The presence of tumour cells in the circulation is necessary, but certainly not sufficient, to produce metastasis<sup>2-5,15</sup>. As discussed later, most tumour cells that enter the circulation do not survive to complete the process.

Knowledge of the expected tissue distribution of metastases arising from primary tumours in different anatomical locations is important to the clinician who must monitor the progress of a cancer patient and plan appropriate therapy. In man, this information has come from descriptive observation of the distribution of metastases arising from particular types of tumours. More accurate information on the behaviour of circulating tumour cells has been obtained in experimental animals by studying the distribution and fate of radiolabelled tumour cells<sup>2</sup>.

## The pathogenesis of a metastasis



**Fig. 1** Schematic illustration of the major steps in the pathogenesis of metastasis. Initial neoplastic transformation of susceptible cells gives rise to a small population of tumour cells. Vascularisation of this early neoplastic lesion allows further proliferation of tumour cells and enlargement of the primary tumour. Malignant cells within the primary tumour next begin to invade the surrounding host tissue(s). Entry of invading tumour cells into lymphatics or blood vessels serves to transport them to distant sites in the body where they lodge and arrest in the capillary beds of various organs. For illustrative purposes this figure depicts arrest of malignant cells in the lung capillary bed. The arrested cells then exit from capillaries into the surrounding lung parenchyma where, subject to provision of a suitable environment, they proliferate to form metastases. A more detailed discussion of each of these steps is given in the text. (Figure reproduced with permission from ref. 103).

Some degree of nonspecific trapping and arrest of circulating tumour cells in the microcirculation occurs simply as a result of mechanical factors. The size and deformability of tumour cells, the diameter and distensibility of capillaries and the interaction of tumour cells with each other and with circulating host cells to create multicellular emboli are all relevant in this context<sup>2-4,10</sup>.

Although mechanical factors undoubtedly exert some effect on the distribution and viability of circulating tumour cells, it is now clear that mechanical factors alone are not sufficient to explain the site(s) of tumour cell arrest and metastasis formation in all human and animal tumours. Clinical observations in man suggest that certain tumours consistently metastasise to particular organs<sup>2,12,16-18</sup>. Non-random patterns of tumour cell arrest and subsequent metastasis formation have also been described in a variety of animal tumour systems<sup>19-26</sup>. Also, experimental studies in which radiolabelled tumour cells are introduced into the circulation have shown that in many instances circulating cells arrest in a wide variety of organs but metastases occur only in certain organs<sup>2,3</sup>. Why some organs support metastatic growth, while others that receive viable tumour cells do not, remains unknown.

Recent evidence indicates that the properties of circulating tumour cells can affect their arrest pattern. A remarkable demonstration of the ability of tumour cells to arrest and grow preferentially in particular organs is provided by experiments showing that animal tumour cells which ordinarily arrest in the lung also localise and grow in pieces of lung implanted subcutaneously (refs 24, 27 and I. R. Hart, unpublished data). In contrast, tumour growth does not occur in subcutaneous implants of other organs. Comparable findings have been obtained in experiments in which the melanoma cells shed from pulmonary metastases in one animal were found to localise preferentially in the lung of non-tumour bearing parabiotic animals<sup>28</sup>.

Equally dramatic evidence of the influence of tumour cell properties on the arrest pattern of circulating cells has come from recent work on the experimental manipulation of plasma membrane composition in B16 melanoma sublines of differing metastatic potential<sup>29</sup>. In these experiments, plasma membrane

components were transferred between different sublines by taking advantage of the fact that B16 melanoma cells growing *in vitro* and *in vivo* spontaneously shed closed vesicles of intact plasma membrane which can be collected and fused with other cells using polyethylene glycol. Membrane vesicles from the B16-F10 subline, which has a high capacity for lung colonisation, were fused with B16-F1 cells, which produce few pulmonary metastases. When the vesicle-modified cells were injected intravenously into C57BL/6 mice they were found to arrest in the lung capillary bed in significantly greater numbers in the lung capillary bed than untreated B16-F1 cells (Fig. 2). Equally important, increased cell arrest resulted in increased production of pulmonary metastases<sup>29</sup> (Table 1). The possibility that the increased arrest of vesicle-modified B16-F1 cells in the lung was due simply to increased cell volume produced by vesicle uptake was excluded by showing that the volume of vesicle-treated cells (determined from cells isolated by centrifugal elutriation) did not differ significantly from control B16-F1 cells<sup>29</sup>.

In related experiments, lymphocyte-resistant sublines of B16-F1 were rendered susceptible to *in vitro* killing by cytotoxic lymphocytes by treatment with vesicles from lymphocyte-sensitive B16 sublines plus polyethylene glycol<sup>29</sup>. Since lymphocyte-mediated cytotoxicity occurs only when the target antigen resides within the plasma membrane<sup>30-32</sup>, this experiment also shows that the vesicles fuse with the cellular plasma membrane and are not merely adsorbed to the cell surface.

The effect of B16-F10 vesicles in modifying the arrest of B16-F1 cells was highly specific. Vesicles from B16-F10<sup>11</sup> cells (a variant of the B16-F10 subline that is resistant to killing by syngeneic immune lymphocytes, but which resembles B16-F1 in its arrest and metastatic behaviour) had no effect on the arrest of B16-F1 cells (Fig. 2). Interestingly, fusion of B16-F1 vesicles with B16-F10 cells did not alter the arrest or metastatic behaviour of these cells (Fig. 2) but this may have been because the vesicles were not introduced in sufficient amounts.

Studies using B16-F1 vesicles surface-labelled with <sup>125</sup>I or ferritin revealed that vesicle components persist in the plasma membrane of B16-F10 acceptor cells for only 18-24 hours<sup>33</sup>.



How then does vesicle treatment enhance metastasis formation when proliferation of extravasated cells to form metastases does not begin until 1–2 days after injection<sup>3,29</sup> when vesicle components are no longer present? Other studies have shown that B16 cells invade the blood vessel wall within 2–4 hours after their arrest<sup>33</sup>. Once within the vessel wall, the tumour cells are less susceptible to damage by haemodynamic forces or circulating host cells and therefore have a greater opportunity of continuing the process of metastasis. Thus, by simply increasing the efficiency of cell arrest and invasion in the lung capillary bed, vesicle-induced modification of B16-F1 cells would increase the likelihood of metastasis formation even though vesicle components *per se* will make no direct contribution to any events occurring 24 hours after initial cell arrest.

Ultrastructural studies indicate that tumour cells adhere to the endothelium and also to the underlying basement membrane<sup>2,13</sup>. Recent studies by one of us (G.P.) suggest that it may be profitable to concentrate further studies on the subendothelial basement membrane of blood vessels since it seems to offer a better substrate for adhesion of malignant tumour cells than does the endothelium (Table 2). Also, tumour cells from clones of a murine fibrosarcoma with high metastatic potential adhere to the endothelial basement membrane more efficiently than clones of low metastatic potential (Table 2).

Following arrest and implantation in the vessel wall, tumour cells must exit from the vessel into the surrounding tissue(s). Extravasation of malignant cells is believed to involve mechanisms similar to those responsible for the initial invasion of blood vessels. Recent observations showing that spontaneously metastatic B16 melanoma sublines produce high levels of type IV collagenase<sup>34</sup> may be particularly significant in this context since type IV collagen is the predominant form of collagen in the basement membrane of capillaries<sup>34</sup>.

Successful extravasation and limited proliferation of malignant cells creates small colonies of tumour cells, so called micrometastases, which at first surround blood vessels<sup>35</sup>. Further growth of these lesions to form macroscopic metastases of clinical importance requires ingrowth of new blood vessels to provide adequate nutrition to support cell proliferation. The ability of certain tumour cells to release a factor(s) that stimulates growth of new blood vessels (angiogenesis)<sup>35</sup> may be of

advantage in this regard. It is important to note, however, that angiogenic factors are also produced by host lymphocytes and macrophages<sup>35</sup>. Release of angiogenic factors from these host cells as a consequence of interaction with metastatic cells, together with release of cell growth stimulating factors by host platelets, could create a situation in which the normal host homeostatic mechanisms increase the risk of metastasis.

## The immune response and metastasis

Throughout the metastatic process, malignant cells are subject to attack by host defence systems, which can be immune<sup>36,37</sup> or nonimmune<sup>38</sup> in nature. Despite an enormous effort to define the role of immune mechanisms in the recognition and destruction of tumour cells, their contribution remains unclear, and convincing evidence that they influence the outcome of neoplastic disease in man is still lacking.

The classic 'immune surveillance' theory<sup>39</sup> attributes host resistance to tumours to the destruction of susceptible tumour cells by mature immune T lymphocytes. This hypothesis, although widely accepted at first, has come under increasing criticism in recent years. The following findings have been cited as evidence against a central role for host immunity in surveillance against neoplastic cells: the incidence of spontaneous tumours in T cell-deficient nude mice is no higher than in immunocompetent animals; most tumours that metastasise in normal immunocompetent animals fail to do so in nude mice; some murine melanoma cells that are resistant to killing by T lymphocytes are less metastatic than cells that are susceptible to killing by T cells; and tumour growth rate in immunologically privileged sites is no faster, and often slower, than that of neoplasms in immunologically exposed sites (see refs 2, 40–44). Also, spontaneous tumours in both man and animals frequently lack detectable antigenic alterations<sup>45,46</sup> and might thus avoid recognition by the immune system. This conclusion comes, however, from experiments designed to detect transplantation resistance and other immune responses involving T cells. The possibility that tumour cells express antigenic or other surface changes recognised by other host defence cells such as natural killer (NK) cells or macrophages still has to be tested (see below).

Experimental studies on the role of host immunity in cancer metastasis have yielded contradictory results. In many transplantable tumour systems, depression of host immunity increases the incidence of experimental and spontaneous metastasis (see ref. 47); in other tumour systems, depression of immunologic reactivity decreases, or even prevents, metastasis<sup>48–51</sup> or has no influence on the growth of a local or disseminated tumour<sup>52</sup>. The basis for these discrepancies could be that the studies were carried out with tumours of various histologic types, of different aetiologies, and induced in different species. We have attempted to minimise these variables by testing three C3H fibrosarcomas with different degrees of immunogenicity for their metastatic activity in immunosuppressed normal, sham-suppressed and immunologically reconstituted syngeneic mice<sup>52</sup>. The highly immunogenic fibrosarcoma formed more pulmonary tumour colonies in immunosuppressed mice than in normal, sham-suppressed (sham-thymectomy and 450R) or reconstituted animals (thymectomy, X-ray irradiation, plus 10<sup>7</sup> normal syngeneic lymphocytes intravenously). A fibrosarcoma of intermediate immunogenicity also formed more pulmonary metastases in immunosuppressed recipients, but this increase could not be reversed by reconstitution with 10<sup>7</sup> normal lymphocytes. In contrast, the least immunogenic tumour formed fewer pulmonary tumour colonies in immunosuppressed mice than in normal, sham-suppressed or reconstituted mice. Thus, even in these relatively uniform conditions, the influence of the immune system on experimental cancer metastasis varied for different tumours. These data also show that tumour immunogenicity is an important factor in the relationship between host immunity and tumour dissemination<sup>52</sup>.

**Table 1** Formation of experimental metastases by B16 melanoma sublines and B16 sublines treated with plasma membrane vesicles

| Cell type | Plasma membrane vesicles | PEG/PHA† | Average number of pulmonary metastases* |
|-----------|--------------------------|----------|-----------------------------------------|
| B16-F1    | None                     | –        | 16 ± 7                                  |
| B16-F1    | None                     | +        | 14 ± 6                                  |
| B16-F10   | None                     | –        | 114 ± 24                                |
| B16-F10   | None                     | +        | 87 ± 16                                 |
| B16-F1    | B16-F10                  | –        | 19 ± 8                                  |
| B16-F1    | B16-F10                  | +        | 44 ± 11                                 |
| B16-F10   | B16-F1                   | –        | 103 ± 16                                |
| B16-F10   | B16-F1                   | +        | 94 ± 19                                 |
| B16-F1    | B16-F1                   | +        | 11 ± 6                                  |
| B16-F10   | B16-F10                  | +        | 91 ± 15                                 |

The B16 mouse melanoma cell sublines were incubated with purified preparations of plasma membrane vesicles from the indicated donor cells in the presence (+) or absence (–) of PEG and PHA for ½ h at 37 °C as described in Fig. 2. Control cell populations which were not treated with plasma membrane vesicles were incubated in serum-free Dulbecco's modified Eagle's medium for ½ h at 37 °C. Vesicle-treated and control cell populations were then tested for their ability to form experimental metastases after i.v. injection into 10-week-old C57BL/6 mice (5 × 10<sup>4</sup> cells per animal) as described in the legend to Fig. 2. Formation of experimental metastases was measured 18 days after injection of cells.

† PEG = polyethylene glycol; PHA = phytohaemagglutinin.

\* Mean values (±s.d.) derived from measurements on 20 mice per group in two separate experiments. (Table derived from data published in ref. 29).

**Table 2** Adhesion of non-neoplastic cells and metastatic tumour cell variants to endothelial cells and subendothelial basement membrane

| Cell                           | Metastatic potential* | % Cell adhesion†   |                                  |
|--------------------------------|-----------------------|--------------------|----------------------------------|
|                                |                       | Intact endothelium | Subendothelial basement membrane |
| C57BL/6 mouse embryo cells     | None                  | 35.6 ± 1.4         | 30.2 ± 1.2                       |
| B16-F1 melanoma                | Low                   | 39.2 ± 1.7         | 46.4 ± 1.4                       |
| B16-F10 melanoma               | High                  | 33.4 ± 0.9         | 69.7 ± 1.5                       |
| B16-F1-BV8 melanoma†           | High                  | 36.1 ± 1.3         | 83.7 ± 2.3                       |
| C3H mouse embryo cells         | None                  | 35.2 ± 0.9         | 31.6 ± 1.1                       |
| UV-2237 fibrosarcoma, clone 15 | Low                   | 27.4 ± 1.1         | 39.3 ± 1.3                       |
| UV-2237 fibrosarcoma, clone 46 | Low                   | 38.7 ± 1.3         | 49.2 ± 1.6                       |
| UV-2237 fibrosarcoma, clone 38 | Low                   | 31.9 ± 1.5         | 48.6 ± 1.5                       |
| UV-2237 fibrosarcoma, clone 34 | Medium                | 39.4 ± 0.9         | 64.2 ± 1.6                       |
| UV-2237 fibrosarcoma, clone 9  | Medium                | 30.7 ± 1.2         | 67.5 ± 2.0                       |
| UV-2237 fibrosarcoma, clone 39 | High                  | 45.2 ± 1.8         | 65.8 ± 1.7                       |
| UV-2237 fibrosarcoma, clone 25 | High                  | 37.4 ± 1.3         | 80.4 ± 1.5                       |

Actively growing monolayer cultures of the indicated cell types were labelled with  $^{125}\text{I}$ -IUdR as described in the legend to Fig. 2. Aliquots containing  $2.5 \times 10^4$  radiolabelled cells were then plated onto the surface of 1 cm<sup>2</sup> segments of human umbilical vein in 0.1 ml Dulbecco's modified Eagle's medium containing 10% calf serum. The radioactivity present in five representative inocula of each cell type was measured in a Packard gamma counter. After addition of radiolabelled cells the blood vessel segments were incubated for 1 h at 37 °C. The culture medium plus non-attached cells was then aspirated and the surface of the blood vessel was washed twice with prewarmed Hanks' balanced saline solution (HBSS). The amount of radioactivity associated with the blood vessel segments was then measured and expressed as a percentage of the total radioactivity in the original inoculum. In assaying cell adhesion to intact endothelium the structural integrity of the endothelium was confirmed by scanning electron microscopy. Vessels showing partial detachment of endothelial cells or regions of complete loss of endothelial cells were excluded from the adhesion assay. For assays of cell adhesion to the subendothelial basement membrane, a balloon catheter was used to remove the majority of endothelial cells after which the vessel was treated with 0.5% EDTA to remove any remaining endothelial cells. Complete removal of endothelial cells was then confirmed by scanning electron microscopy.

\* Ability to form experimental pulmonary metastases after i.v. injection of  $1 \times 10^5$  cells. The classification refers to median number of experimental metastases produced in 10 animals<sup>70,71</sup>: low (1–15); medium (30–50) and high (>100).

† A variant isolated from the B16-F1 subline by *in vitro* selection for enhanced ability to invade the wall of blood vessels maintained *in vitro* in a perfusion culture system<sup>78</sup>.

‡ Mean ± s.e.m.

The existence of a system of natural host defence against tumour cells mediated by natural killer (NK) cells<sup>42</sup> offers a possible alternative mechanism for cell-mediated surveillance against circulating neoplastic cells. The role of NK cells, and also activated or armed macrophages<sup>53</sup>, in limiting tumour growth and metastatic spread, as well as the related questions of amplifying NK- and macrophage-mediated destruction of tumour cells<sup>54,55</sup> as methods for treating micrometastases are presently being studied in many laboratories and the outcome of this work is awaited with considerable interest.

## Metastasis as a selection process and variation in the metastatic capabilities of tumour cells

Metastasis is a highly selective process. The malignant cells that complete all of the steps described in the previous section and establish metastases have survived a demanding sequence of

hostile events which destroy the majority of tumour cells<sup>2–4</sup>. For example, only a small proportion of the total cells from the primary tumour may gain access to the circulation and of these as few as 0.1% may survive the trauma of dissemination and arrest<sup>2</sup>. Surviving cells suffer further loss during extravasation and the viability of extravasated cells, in turn, may depend on the availability of appropriate nutrients and escape from destruction by host defence systems.

Recognition that only very few tumour cells survive to develop into metastases raises the important question of whether survival is random or non-random. Random survival would mean that the primary tumour is populated by cells of equal metastatic potential, any one of which could give rise to a metastasis. The alternative possibility is that metastasis is not random, but involves the survival of specialised tumour cells endowed with specific properties that befit them to complete each step in the metastatic process. In this case, the primary tumour would contain subpopulations of cells of differing metastatic potential, including cells with little or no capacity to metastasise.

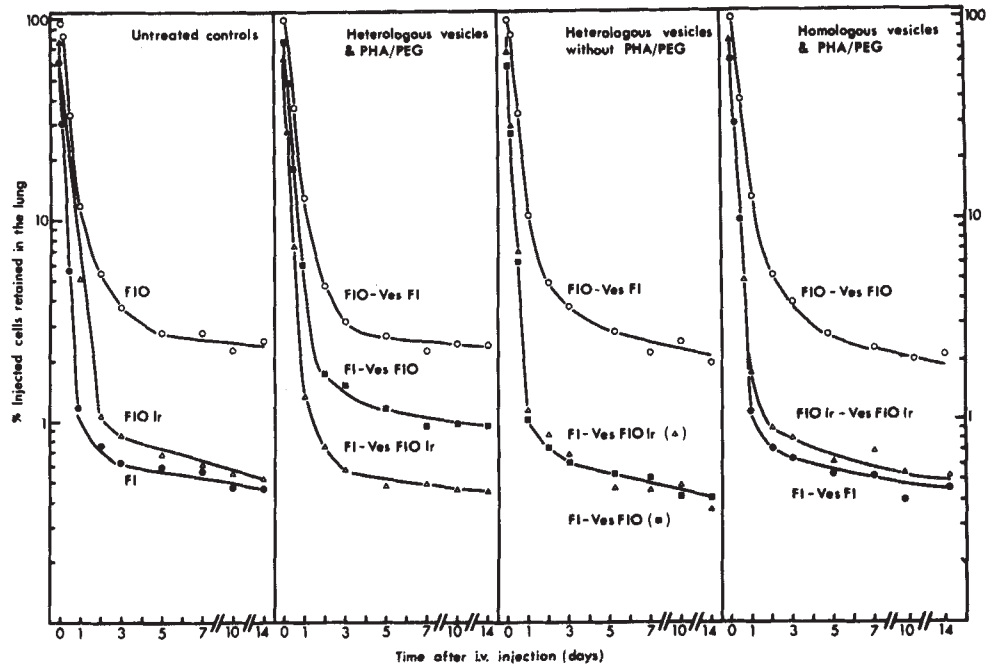
The possibility that cells with high metastatic potential can be isolated from a heterogeneous parental tumour by selection procedures was first suggested in 1939 by Koch who isolated a highly metastatic subline from the Ehrlich carcinoma tumour by serially transplanting lymph node metastases<sup>56</sup>. In 1955, Eva Klein demonstrated that gradual conversion of some solid murine neoplasms into ascites variants was due to the selective overgrowth of a small number of cells which differed from the parental population in their ability to proliferate in the peritoneal cavity and metastasise to the lungs<sup>57</sup>. Since the change was stable and heritable, Klein concluded that the gradual conversion of the solid tumour to the ascites form involved mutation/selection and not adaptation. Further evidence of heterogeneity in the metastatic potential of tumour cells has come from more recent experiments in which selective harvesting of cells from metastases during successive passages of tumour cells *in vivo* has yielded cell populations with a greater metastatic potential than cells from the original cell population<sup>19,22,58–70</sup>. In this type of *in vivo* selection the isolated cell populations are not cloned, and the enhanced metastatic potential detected in the population as a whole is due to enrichment of the proportion of highly metastatic cells in the population at each successive passage.

Definitive evidence that malignant primary tumours contain subpopulations of cells with differing metastatic capabilities was obtained in 1977 by Fidler and Kripke<sup>70</sup> using the B16 melanoma syngeneic to the C57BL/6 mouse. To investigate whether primary tumours contained cells of differing, or uniform, metastatic potential they prepared a cell suspension from a subcutaneous primary tumour and divided it into two aliquots. One part was immediately assayed for its ability to form experimental pulmonary metastases following intravenous injection (a procedure for assaying cellular metastatic ability which has been validated elsewhere<sup>71</sup>). From the second part of the original suspension, 17 clones were isolated and their progeny tested for their ability to produce experimental metastases. If the tumour was populated by cells of uniform metastatic potential, then the cloned sublines should each produce the same number of metastases as the uncloned parental population. This was not the case. The original uncloned parental tumour cell population produced similar numbers of metastases in different animals but the cloned sublines differed markedly in their metastatic potential (Fig. 3). Although the number of metastases produced by any given clone was constant, individual clones showed wide variation in their metastatic potential. Control subcloning experiments demonstrated that the cloning process was not responsible for the variability<sup>70</sup>. These experiments thus suggest that the parent B16 tumour is heterogeneous and that highly metastatic tumour cell variants preexist in the parental population<sup>70</sup>.

To exclude the possibility that the metastatic heterogeneity found in B16 melanoma clones might have been introduced as a



**Fig. 2** Lung arrest and retention of  $^{125}\text{I}$ -IUdR labelled B16 melanoma sublines and B16 sublines treated with plasma membrane vesicles after i.v. injection into C57BL/6 mice. Plasma membrane vesicles (Ves-F1; Ves-F10<sup>lr</sup>; Ves-F10) were purified from the culture medium of actively growing roller bottle cultures of the various B16 sublines and were purified as described elsewhere<sup>29</sup>.  $^{125}\text{I}$ -IUdR-prelabelled cells from the indicated B16 sublines (F1, low lung metastasis; F10, high lung metastasis; F10<sup>lr</sup>, lymphocyte-resistant variant of F10 with low metastatic capacity) were incubated in suspension with homologous or heterologous vesicles (300–400  $\mu\text{g}$  protein per  $10^7$  cells) in serum-free Eagles minimum essential medium (MEM) with or without 50  $\mu\text{g ml}^{-1}$  PHA-P and PEG 6000 as described elsewhere<sup>29</sup>. Control cells were incubated without vesicles in serum-free MEM for the same period. Vesicle-treated and control cells were then washed three times with prewarmed Hanks' balanced saline solution (HBSS) and incubated for 10 min at 37 °C in 0.2% EDTA to separate multicellular clumps into single cells. Since cell size can influence cell arrest in the microcirculation, vesicle-treated and control cells injected into animals (see below) were subjected to density gradient centrifugation and centrifugal elutriation to obtain cell fractions of similar size and density<sup>29</sup>. Aliquots of  $1 \times 10^5$  viable, radiolabelled cells with a modal volume in the range  $1.4\text{--}1.6 \times 10^3 \mu\text{m}^3$  were then injected as a single cell suspension in 0.2 ml HBSS into the tail vein of unanaesthetised C57BL/6 mice. The radioactivity present in five representative inocula was monitored for each cell preparation in a gamma counter. Lungs were collected from injected mice at intervals from 5 min to 14 days after injection and placed in vials containing 70% ethanol. The ethanol was changed daily for 3 days to remove alcohol-soluble radioactivity. The remaining lung-associated radioactivity represents radioactivity incorporated into the DNA of tumour cells that were viable at the time the animals were killed<sup>104</sup>. Lung-associated radioactivity at the indicated times after injection is expressed as a percentage of the injected cells retained in the lung. Each point represents a mean value derived from measurements of five separate animals. The arrest kinetics of  $^{125}\text{I}$ -IUdR-labelled F1, F10 and F10<sup>lr</sup> cells treated with PHA/PEG but not exposed to vesicles did not differ from that shown in the left-hand panel for untreated control cells from these sublines. Figure constructed from data given in ref. 29.



result of the lengthy *in vivo* and *in vitro* cultivation that this tumour has undergone since its isolation in 1954, Kripke and Fidler repeated the same experiment with a newly induced primary tumour and tested the metastatic potential of cells isolated from this tumour after only 6 passages *in vitro*. The results obtained with this tumour, a UV-induced fibrosarcoma (UV-2237) of a C3H mouse, were similar to those obtained with the B16 melanoma. Similarly, marked heterogeneity in metastatic potential has now been demonstrated in cloned populations isolated from a murine mammary tumour<sup>72</sup>, a murine MCA-induced fibrosarcoma<sup>73</sup>, a murine sarcoma virus-transformed fibrosarcoma<sup>26</sup> and a transformed rat liver epitheloid cell line<sup>74</sup>. More recently, we have shown that whereas clones isolated from the primary tumour show widely differing metastatic abilities, cloned sublines isolated from individual metastases exhibit a much more uniform metastatic potential. This suggests that metastases, in contrast to the primary tumour, are more homogeneous and populated by cells with similar metastatic capabilities (Fig. 3).

Metastatic and non-metastatic tumour cell variants derived from the same parent tumour cell population have also been isolated using *in vitro* selection methods in which cell populations are selected for expression (or failure to express), a property considered of possible importance in one or more steps in the metastatic process. The metastatic behaviour of such isolated cells is then assayed *in vivo* to determine whether the particular selection has enriched the population for cells with increased or decreased metastatic capacity. Selection *in vitro* for detachment from a monolayer<sup>75</sup>, resistance to lysis by lymphocytes<sup>76</sup>, resistance to lectin-mediated toxicity<sup>77</sup>, attachment to

collagen<sup>58</sup> and ability to invade various tissues maintained in organ culture<sup>78</sup> have now been used successfully to isolate tumour cell lines with increased or decreased metastatic capabilities.

Collectively, this large body of evidence indicates that malignant tumours are not uniform entities populated by cells of equal metastatic potential. Rather, primary tumours are populated by subpopulations of cells of widely differing metastatic potential. This conclusion supports the proposal made by Nowell<sup>79</sup> that during tumour progression variant cells arise which are subject to host selective pressures that favour the survival of variants with increased malignant capacity. Regardless of whether neoplasms are monoclonal or multiclonal in origin<sup>79,80</sup>, by the time they can be diagnosed clinically they could be heterogeneous and contain subpopulations of cells with a wide range of phenotypic characteristics. Recent studies by one of us (I.J.F.) using mouse fibroblasts transformed *in vitro* by cloned stocks of murine sarcoma virus have shown that extensive heterogeneity in metastatic potential is present in subclones derived from the original transformed cells as little as 28 days after the initial transformation.

Heterogeneity in the metastatic potential of cells in the primary tumour is perhaps not unexpected. Cells populating both human and animal neoplasms are known to be heterogeneous with respect to their immunogenicity<sup>83</sup>, antigenic properties<sup>82–84</sup>, growth rate<sup>85</sup>, metabolic characteristics<sup>86</sup>, hormone receptors<sup>87</sup>, pigment production<sup>88</sup>, radiosensitivity<sup>89</sup> and susceptibility to cytotoxic drugs<sup>90–92</sup>. Of even more interest in the context of the present discussion are recent observations showing that tumour cells in primary and metastatic lesions

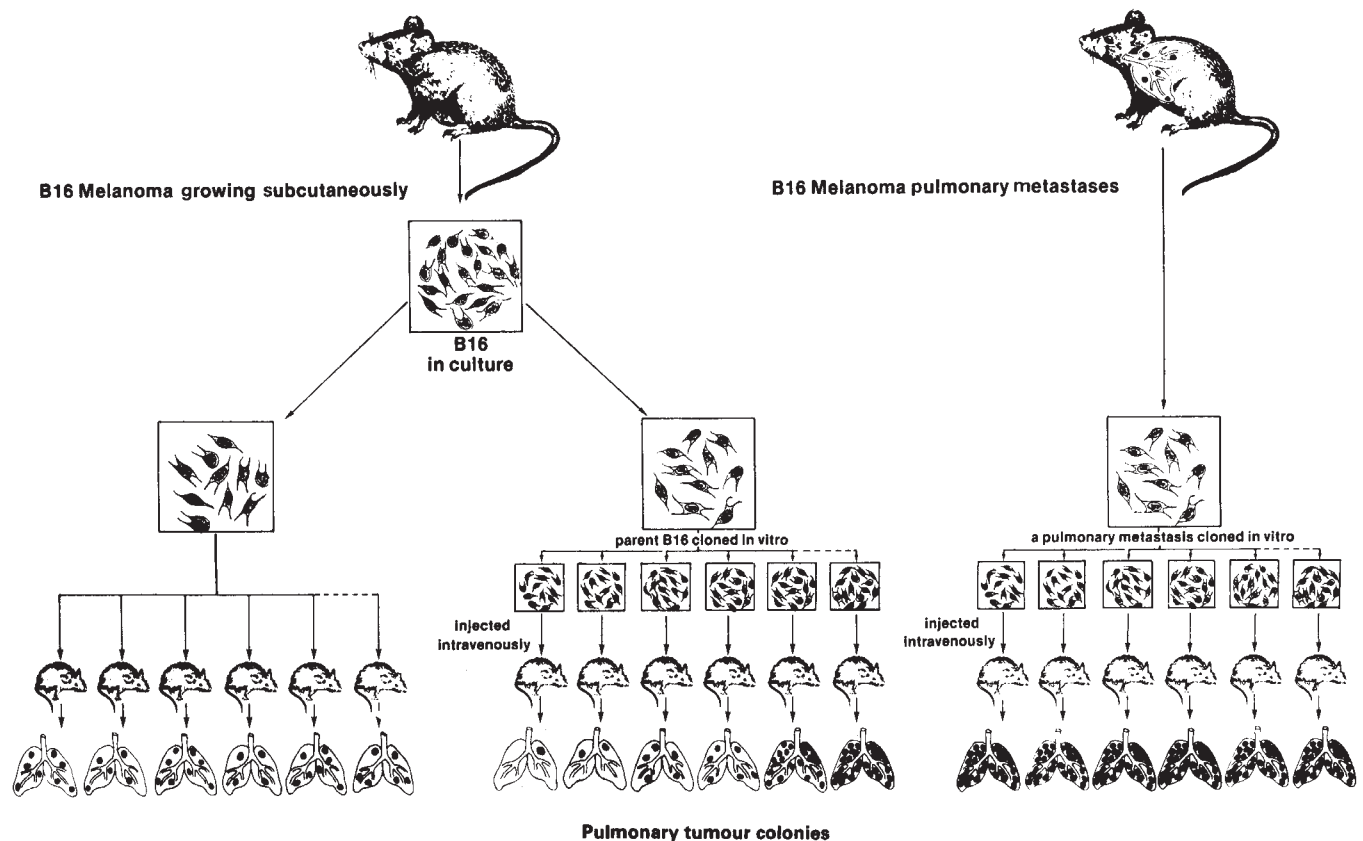
differ in their antigenic properties<sup>93</sup>, biochemical characteristics<sup>94</sup> and response to cytotoxic drugs<sup>61,95-97</sup>

## Implications

The presence within the primary tumour of subpopulations of cells with differing metastatic capabilities has profound implications for the way in which research is done to identify the cellular properties responsible for metastasis. Hitherto, the general experimental approach to this problem has been to study cell populations isolated at random from the primary tumour and to assume that properties detected in these cells are also expressed by cells that give rise to metastases. The demonstration of metastatic heterogeneity in the cells of the primary tumour indicates that this assumption is not valid. Experimental efforts to identify features unique to metastatic subpopulations will require isolation of such subpopulations and comparison with nonmetastatic (but tumorigenic) subpopulations of cells from the same parent tumour. Continued efforts to study the properties of malignant cells using unselected heterogeneous cell populations will probably be of little value since the proportion of nonmetastatic or poorly metastatic cells may be sufficiently high to mask properties peculiar to small sub-

population(s) of highly metastatic cells. Moreover, studies that utilise tumour cells isolated from tumours whose detailed origins are not known or tumours propagated by serial transfer intraperitoneally may be unproductive in that the former may not be syngeneic to the recipient animals while the latter may represent a selected subpopulation of cells since growth intraperitoneally has been shown to be selective<sup>57</sup>.

The existence of specialised subpopulations of cells with high metastatic potential has equally far reaching consequences for the testing of potential agents for treating metastatic disease. Both human and animal neoplasms contain subpopulations of cells with differing sensitivities to cytotoxic drugs<sup>90-92</sup>. Differences in the response of primary and metastatic lesions to therapeutic agents is well documented in clinical practice<sup>98</sup> and metastatic cells showing increased resistance to chemotherapeutic agents have been selected in experimental animal tumours<sup>61</sup>. As pointed out recently by Schabel *et al.*<sup>98</sup>, most of the methods presently used to define the drug sensitivity of experimental animal tumours might be misleading since they are based on the belief that transplantable tumours are homogeneous, that is, uniform entities. Assays of the antitumour activities of therapeutic agents that rely on partial regression of the primary tumour may be inadequate for screening agents for



**Fig. 3** Schematic illustration of experiments demonstrating heterogeneity in the metastatic capabilities of clones derived from cells in a parental murine tumour<sup>70</sup> and the relative homogeneity of the metastatic potential in clones derived from cells isolated from a pulmonary metastasis (G.P. and I.J.F., unpublished data). The experiment was similar in design to the classical fluctuation test devised by Luria and Delbruck to distinguish between selection and adaptation in the origin of bacterial mutants<sup>105</sup>. Cells isolated from the parental tumour were divided into two parts. One part (left-hand side of figure) was injected i.v. into 60 C57BL/6 mice to assay formation of experimental pulmonary metastases. The other part (central portion of the figure) was used to produce a series of clones and the progeny of each clone was injected i.v. (10 mice per clone) in an identical procedure to that of the uncloned parental tumour cells. Mice were injected i.v. with  $5 \times 10^4$  viable cells and the number of lung metastases was measured 18 days later by examining the lungs with a dissecting microscope<sup>70</sup>. Cells of the parental population produced a median of 40 lung metastases (range 8–131 metastases). In contrast, cells of clones from the parental tumour differed markedly in their metastatic capabilities, from very low (range of median number of metastases from 7 clones = 3–20), medium (medians from 3 clones = 36–99 metastases), high (medians from 5 clones = 150–260 metastases) to very high metastatic capacity (medians from 2 clones = >500 metastases)<sup>70</sup>. In contrast, clones derived from cells isolated from a single pulmonary metastasis (right-hand side of figure) were of uniform metastatic potential when assayed in similar experimental conditions. This indicates that the metastatic lesion is composed of a more homogeneous cell population with a relatively uniform metastatic capability. The clones shown in the figure produced a uniformly high number of metastases. Although the number of metastases produced by cells isolated from any given metastasis is uniform, clones isolated from different metastases may differ substantially in the number of metastases they produce (G. P. and J. Doll; I. J. F., E. Gruys and M. L. Kripke, unpublished data). This reflects differences in the metastatic activities of the original tumour cell subpopulations residing within the parental tumour which give rise to individual metastases<sup>70</sup>.



their antimetastatic activity. Assays in which a drug, or combination of drugs, are found to limit growth of the primary tumour to 50% of that in untreated controls may merely reveal the presence of a subpopulation(s) of drug-sensitive cells and do not necessarily offer any insight into the pharmacologic susceptibility of the tumour cell subpopulations that will give rise to metastases. Future efforts to identify potential agents for treatment of metastases might thus more profitably adopt assays in which therapeutic agents are screened for their activity against cloned tumour cell subpopulations with defined metastatic capabilities.

Phenotypic heterogeneity is not only a problem for the chemotherapist. Immunologic heterogeneity among tumour cells<sup>82-84, 93, 99</sup> poses equally serious problems for efforts to treat neoplastic disease by specific immunotherapy<sup>100</sup>. For example, analysis of a number of AKR mouse lymphomas of recent origin has shown that these tumours are immunologically polyclonal, consisting of a numerically dominant subpopulation and several distinct minor subpopulations that comprise less than 3% of the tumour. Attempts to induce tumour rejection by immunising tumour-bearing animals using unfractionated thymoma cells were unsuccessful since effective immunisation occurred only against the dominant clone and the minor subclones did not offer a sufficient immunologic mass to stimulate the immune response<sup>101</sup>. Immune rejection of the dominant clone merely permits the minor subpopulations to proliferate and become dominant. This situation is analogous to the well documented problem of drug-resistance in cancer chemotherapy where resistant variants preexist within the parental tumour population, albeit as a minor subpopulation, but following destruction of the original dominant drug-sensitive population(s) they proliferate unchecked<sup>102</sup>.

Recognition that primary malignant tumours are not populated by cells of equal metastatic potential, but contain subpopulations of cells with widely differing metastatic capabilities, calls for critical reappraisal of the adequacy of many of the experimental systems presently used in studying 'malignancy' and in screening agents for treating metastatic disease. Experimental systems that fail to take account of the heterogeneity of metastatic potential of neoplasms may prove to be less successful in providing insight into the basic mechanisms involved in metastasis than systems that embrace this concept. We thus consider the isolation and characterisation of tumour cell variants of defined metastatic potential from human and animal neoplasms to be of major importance for future progress in metastasis research.

The search for a property uniform to all metastatic cells may be unproductive. Work carried out with isolated variant tumour cell lines must consider that the development of metastases is dependent on an interplay between host factors and the intrinsic properties of the tumour cells. To establish metastases, tumour cells must complete *all* steps involved in the metastatic process. Enhanced performance of a tumour cell in one step of the process cannot compensate for an inability to complete a subsequent step. The failure of most tumour cells to produce metastases need not be due to a single, common factor. Interruption of the metastatic sequence at any step in the process will prevent the production of clinical metastasis. On a more optimistic note, the increasing availability of tumour cell populations with differing metastatic capabilities should facilitate experimental efforts to identify the properties which enable malignant cells to metastasise. With the availability of this knowledge it might then be possible to design more effective procedures for the treatment of this catastrophic disease.

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## ARTICLES

# New results from optical polarimetry of Saturn's rings

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*Linear polarimetry of Saturn's rings, obtained through the period of the 1979 opposition, is presented. The polarisation clearly correlates in direction with the plane containing the Sun, planet and Earth, but not the ring plane. The results are consistent with local scattering on the surface of individual ring bodies, covered with frost.*

SATURN'S rings have intriguing and rather special optical properties. The rings undergo a sharp surge in brightness during a narrow phase interval around opposition<sup>1</sup>, when the Sun, Earth, and Saturn are aligned. Water ice in some form is present on the rings and apparently controls their optical behaviour<sup>2,3</sup>. The geometrical arrangement of the ring particles—either a single monolayer of bodies or a thicker (low-density) disk—has long been a subject of speculation<sup>4</sup>. The typical size of a ring body is poorly known, with diameter estimates ranging from 1 cm to 1 km (ref. 5). One Earth-based tool which is useful for probing the nature of the rings is the measurement of the polarisation of light reflected from the rings.

### Polarisation theory

The laboratory investigations of Lyot<sup>6</sup>, Dollfus<sup>6,7</sup>, and others have provided much information on the polarisation of light reflected from terrestrial and lunar materials. These studies have shown that the variation of polarisation with phase angle (angle between the incident and emergent light rays) is diagnostic of both the bulk properties and the texture of the surface material.

The polarisation of light reflected from a flat, polished surface is polarised so that the electric vector (**E** vector) of the polarised component is parallel to that surface, in compliance with Fresnel's laws of reflection. Typical Solar System bodies, however, tend to be covered by either a particulate or rough and pitted surface, presenting an ill-defined surface boundary. The light scattered from such a surface is found to be polarised with the electric vector lying either in the scattering plane (termed negative polarisation) or perpendicular to that plane (positive polarisation), depending on the phase angle and other parameters. The so-called negative branch is confined to a narrow interval around zero phase, that is, around opposition.

On the grounds of general symmetry, we might expect two types of contributions to the polarisation of Saturn's rings, one correlated with the ring plane, the other with the scattering plane (the plane including the Sun, planet and Earth). The idea of a ring-plane polarisation is particularly attractive because Saturn's rings are extremely thin and flat within telescopic resolvability; and physical mechanisms can easily be cited for such an effect. One characteristic of this type of polarisation, correlated in direction (position angle) with the ring plane, is that it should not vanish at precise opposition. It would be seen

most clearly at opposition, as other polarisation effects correlated only with the scattering plane would then vanish, the scattering plane being undefined at zero phase. We will show that the absence of correlation with the ring plane means that any polarising mechanism related to the ring plane, as such, must be ineffective.

Only a few investigations of the linear polarisation of the light reflected by Saturn's rings have been published since Lyot's original discovery in 1923<sup>6–10</sup>. Lyot<sup>6</sup> attempted to explain the polarisation of the rings as being due to two or three independent mechanisms: (1) direct scattering of sunlight by individual ring bodies; (2) scattering of light from Saturn's disk, by the rings; and (3) multiple scattering between aligned ring bodies. Mechanisms (2) and (3) would produce a polarisation of the type with a position angle related to the ring plane. Dollfus<sup>9</sup> finds a component in the polarisation of the B ring with a position angle directed either radially or tangentially, relative to the centre of Saturn, unexpectedly varying in time (on a time scale as short as several days). He reports that this rapidly variable component can be as large as 0.2%. Our measurements, with those in ref. 8, show no evidence for such a component. Rather, we find that polarisation varies smoothly with phase angle.

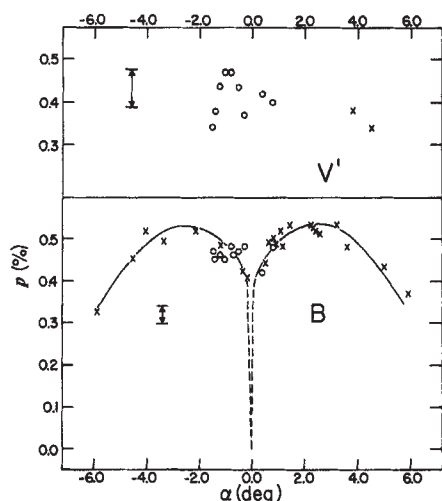
### Observations

Figures 1 and 2 show the linear polarimetry of Saturn's rings obtained between December 1978 and May 1979. The data consist of two independent subsets, acquired at different observatories, each with a separate polarimeter and observing team. Measurements at the Pine Mountain Observatory of the University of Oregon (PMO) were made (by J.C.K., T.E.P. and M.S.B.) using the 61-cm telescope. A 41-cm telescope was used at the Kitt Peak National Observatory (KPNO) (by P.E.J. and R.K.). All PMO observations used a 7 arc s aperture, while at KPNO an 11 arc s aperture was used. Measurements were made with either a standard B filter or a yellow interference filter (*V'*) having  $\lambda_{eff} = 5,530 \text{ \AA}$  and  $\Delta\lambda = 700 \text{ \AA}$ .

Both photoelastic-modulator polarimeters have been checked (in connection with other work) against both polarised and unpolarised standard stars; each has a typical<sup>11</sup> measured instrumental polarisation  $\leq 0.01\%$ . Excellent agreement between the two subsets of data (better than  $\pm 0.02\%$  in  $\Delta P$  for the B-filter data, and  $\pm 2^\circ$  in  $\Delta\theta$ ) is evident in Figs 1 and 2.

All measurements were made on the east-ring ansa, such that the tip of the A ring was approximately centred in the aperture. Thus, portions of both the A and B rings were included in the aperture, with A contributing about twice as much as B to the measured flux, at this inclination of the ring plane relative to the line of sight. Sky measurements were made by offsetting the aperture to the north, by about one aperture diameter, around the arc of a circle centred on Saturn's disk. Even on nights of





**Fig. 1** Polarisation of Saturn's rings in B and V' filters versus solar phase angle ( $\alpha$ ).  $\times$ , PMO data;  $\circ$ , KPNO data. Error bars at the left represent the typical error of one observation. A curve, symmetric about the origin, is hand drawn through the B-filter data. The dashed lines indicate an hypothesised polarimetric opposition effect. The position angle of polarisation always lies in the scattering plane, thus all the  $p$  values are 'negative'.

worst seeing, scattered light so measured never amounted to more than 10% of the flux on the ansa, either in terms of the polarised or total fluxes. Thus, stray light from Saturn's disk or other sources had no appreciable effect on the results.

Figures 2 and 3 show that the angle of polarisation always lies in the Sun-planet-Earth (SPE) plane. Because Saturn's orbital plane is slightly tilted out of the ecliptic plane, near opposition the SPE plane rotates by almost  $180^\circ$  on the plane of the sky. The polarisation follows the SPE plane, rotating also by almost  $180^\circ$ —see Fig. 3: this is our key result.

The tight correlation of the position angles with the position of the SPE plane, and the smoothness of our polarisation-phase curve over weeks, do not suggest any randomly varying component in the B-band polarisation as large as 0.10%. J.C.K. and Murphy<sup>8</sup> did not detect either azimuthal or temporal variations in the angle of polarisation around either the A or B rings to within  $\pm 4^\circ$ . Comparing our measurements (at a ring tilt of  $-6^\circ$ ) with these (at a ring tilt of  $-26^\circ$ ), we also find no evidence for variation of the B-band polarisation with ring tilt, to within  $\Delta p = \pm 0.1\%$ , if comparison is made at the solar phase angle of  $5.5^\circ$ . The polarisation seems to depend only on the SPE (solar-phase) angle, at least in blue light.

## Discussion

Because the angle of polarisation coincides strictly with the SPE plane, the polarisation seems to be mainly generated by intraparticle scattering (local scattering on individual, rough-surfaced ring bodies) and not by interparticle scattering (between discrete ring bodies).

Two other proposed mechanisms for the polarisation of Saturn's rings cannot explain the position-angle variation: (1) light received from the disk of Saturn and subsequently scattered towards us by the rings, or (2) multiple scattering between ring bodies, particularly in a monolayer.

Mechanism (2) here would be analogous to the mechanism which generates the phase-independent 'limb' polarisation which occurs on Jupiter's disk<sup>10</sup>. In that case the polarisation arises from double scattering between aerosol particles in a horizontal thin layer in the jovian atmosphere. This effect is shown in Fig. 4.

In a monolayer, a fraction of the incident sunlight is scattered between ring bodies in paths which lie parallel to the ring plane. Light could be scattered by one ring body (O) to bodies in front of or behind O, that is,  $a$  or  $a'$ ; or to bodies on either side, that is,

$b$  or  $b'$ . But scattering to bodies above or below the ring plane (as  $c$  or  $c'$  in Fig. 4) is absent. This asymmetry results in a net polarisation, even with exact back-reflection, that is, at opposition. The dominant contribution to the polarisation is from the paths from O to  $b$  and  $b'$ , which involve scattering angles of the order of  $90^\circ$  and thus large polarisations. As the polarisation at such angles for solid bodies has a positive sign, the resulting polarisation for the monolayer would no doubt be positive, with the emergent E vector normal to the monolayer.

In a low-density ring (many bodies thick), light paths  $c$  and  $c'$  in Fig. 4 are not eliminated. Then the interparticle scattering will tend to be isotropic, and the process just discussed would be suppressed.

We can set an upper limit to the polarisation contributed by double scattering between ring bodies in a monolayer model by assuming the rings to be a close-packed array of spheres, appropriate for the B ring which was an optical depth close to unity<sup>4</sup>. We write

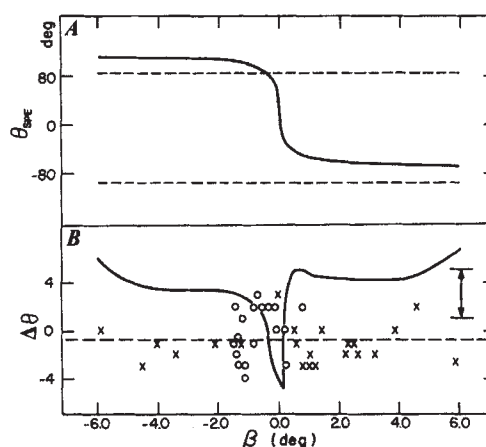
$$p \approx GL \quad (1)$$

where  $G$  is the ratio of doubly-to-singly scattered light, and  $L$  is the polarisation of light reflected by an individual ring body at a scattering angle  $\sim 90^\circ$ . Higher-order scattering is ignored.  $G$  can be viewed as simply that portion of the sunlight impinging on one ring body which is intercepted by a second body and which, also, can then be deflected towards the Earth without shadowing. For an isotropic scattering phase function we find:

$$G \approx a\tilde{\omega}/(4\pi d^2) \quad (2)$$

where  $\tilde{\omega}$  is the equivalent single-scattering albedo for a ring body,  $d$  is the mean length of the scattering path between two ring bodies, and  $a$  is the mean area of all other ring bodies 'seen' by both the initial scatterer and the observer, taking account of the blocking by other ring bodies.  $d = D - R$ , where  $D$  is the mean centre-to-centre separation of ring bodies and  $R$  is the mean ring-body radius.

For the data presented here, the rings are seen close to edge-on. For a close-packed monolayer, only the tops of the individual bodies can be seen from the Earth or the Sun. The rays involved are those striking the top or 'cap' of one sphere, then being scattered by the exposed cap of an adjacent sphere, or more precisely by half of such an exposed cap. From a careful consideration of this geometry we find that at a ring-tilt angle of



**Fig. 2** A, The variation of the position angle of the Sun-planet-Earth plane ( $\theta_{SPE}$ ) with  $\beta$ , the angular distance of Saturn from the sub-solar point, along the ecliptic. (Note that  $\beta \approx \alpha$ , where  $\alpha$  is the Sun-planet-Earth angle, except at very small  $\alpha$ .) The dashed lines indicate the geocentric position of the major axis of the rings. B, The deviation of  $\theta$  (the polarisation position angle) from  $\theta_{SPE}$  (where  $\Delta\theta = \theta - \theta_{SPE}$ ).  $\times$ , PMO data;  $\circ$ , KPNO data. The solid line is the combined result of the measured mean curve (dashed line) with addition, in Stokes-vector space, of a hypothesised phase-independent polarisation of 0.08% having its E vector normal to the ring plane.

6°, with  $\tilde{\omega} = 0.75$ , the parameter  $G$  is quite small, about 0.01. This applies to the B ring. For the A ring, the ring optical depth being less by a half, the  $G$  factor would be also halved.

Thus the effect of a possible 'monolayer' constituent in our observed polarisation can be dealt with if we retain only  $L$  (the polarisation of a ring body at scattering angles  $\sim 90^\circ$ ) as an unknown parameter. A monolayer polarisation would be essentially phase-independent; the  $E$  vector would remain normal to the ring plane and would not follow the rotation of the SPE plane on the sky, as in Fig. 3. The observed angle behaviour is more consistent with a constant  $\Delta\theta = 0$ , that is, with the dashed line at the bottom in Fig. 2. The addition of a small monolayer effect would be to shift the curve  $\Delta\theta$  from the zero line—we would find a perturbed curve  $\Delta\theta(\alpha)$ . Such a function is plotted (solid curve) in Fig. 2, for an arbitrary choice of  $L = 8\%$ . From least-squares fitting of  $\Delta\theta(\alpha; L)$  to our position-angle data, we find that the  $3\sigma$  upper limit for a monolayer-like polarisation is 0.03%. With  $G = 0.01$  as estimated above, we deduce that the  $90^\circ$  polarisation by an individual ring body could not exceed 3%—if the rings were indeed a monolayer.

A similar disturbance  $\Delta\theta(\alpha)$ , (a variable change in position angle away from the SPE plane) would arise from a polarisation constituent due to light from Saturn's disk, rescattered to Earth by the rings. To discuss this we relax the assumption of a monolayer for the rings. Equation (1) applies, but  $G$  is now the ratio of sunlight scattered first by Saturn's disk and then by the rings, to sunlight returned directly to Earth by the rings. A fair value for  $G$  is  $\sim 0.01$ , as has been computed by Price and Baker<sup>13</sup> in another context. This polarisation would again be almost phase-independent, with the emergent  $E$  vector most likely normal to the ring plane. For estimating the magnitude of this effect we assume only single scattering of light by individual ring bodies; we neglect multiple light paths between discrete ring bodies. (Multiple scattering locally on the surface of a rough-surfaced ring body, however, is not ignored; that is included by specifying a value of  $L$  for a typical, macroscopic ring body.) Following equations of Cook *et al.*<sup>14</sup>, we find that the ratio of multiply-to-singly scattered radiation from the rings, in this sense, does not exceed 0.1, for a low-density ring and a single-scattering albedo of 0.75; single scattering by the ring bodies is dominant. As for the pure monolayer effect discussed above, we are again able to place a  $3\sigma$  upper limit on the large-angle ring-body polarisation of  $\sim 3\%$ ; for a larger value, we would surely see a distortion in the  $\Delta\theta$  curve.

Note that, regardless of the precise structure of the rings, our phase curves—especially of the position angle—provide an extrapolated upper limit for the polarisation, at angles of the order of  $90^\circ$ , for scattering from the ring bodies, namely about 3%. The small large-angle polarisation, the moderately large negative polarisation at small angles ( $\sim 0.5\%$  in the blue), and

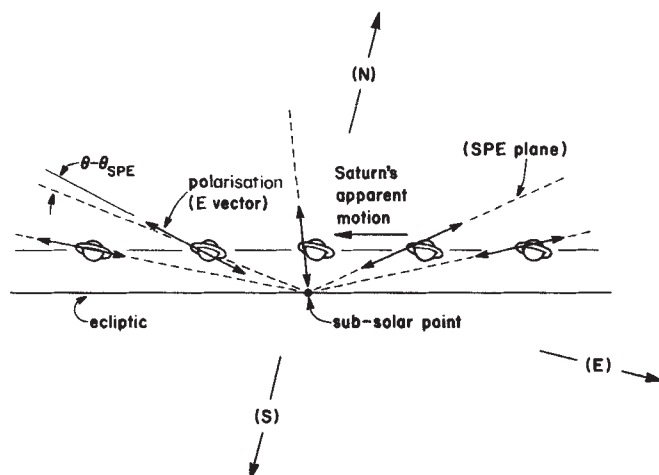


Fig. 3 A representation of the relationship between the position of Saturn's rings and the position angle of the ring polarisation. (Not drawn to scale.)

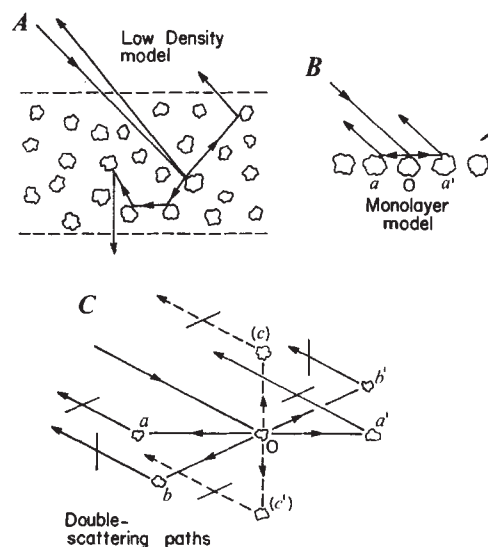


Fig. 4 The low-density and monolayer ring models showing the possible distribution of multiple scattering paths. In C particles  $a$  and  $a'$  are 'in front of' and 'behind' particle O, respectively. Particles  $b, b'$  are 'off to the side'; particles  $c, c'$  are 'above' and 'below' particle O respectively. In a monolayer,  $c$  and  $c'$  are absent. A monolayer viewed from the side is seen in B.

the flatness of  $p(\alpha)$  near opposition, are unique. Only the published polarimetry data of a few laboratory samples are similar. They include a ground, transparent, amorphous silicate, uncontaminated by low-albedo material (contaminated silicates are not similar)<sup>15</sup>, and fine-grained water frost as well as freshly chipped white clay<sup>16</sup>. Thus the polarisation is particularly consistent with frost.

Both experimental evidence<sup>7</sup> and theoretical analysis<sup>17</sup> indicate that negative polarisation ( $E$  vector parallel to the scattering plane) cannot be generated by scattering between widely-separated particles. The polarisation phase curve of Saturn's rings is therefore apparently accounted for strictly by local scattering on individual rough-surfaced bodies, as with the Moon's polarisation; it is not controlled by effects of mutual shadowing and reflection between ring bodies.

Simple rough-surface polarisation should drop to zero at zero solar phase angle. Our phase curves, however, show only a slight trend to zero near opposition, even down to the minimum observed phase angle of  $0.22^\circ$ . The drop to zero must occur in a narrow 'cusp'  $< 0.5^\circ$  wide, for visible wavelengths. This very sharp opposition effect can also be seen in data for both the rings and for high-albedo laboratory samples, reported by Dollfus<sup>16</sup>.

This behaviour agrees qualitatively with the theory of Wolff<sup>17</sup> for the polarisation of light reflected from rough surfaces. In his theory the small-angle polarisation is governed by double reflections between grain facets, combined with shadowing. He predicts a 'cusp' in the polarisation phase curve with a width of the order of that of the photometric ('brightening') effect at opposition. The known width of the latter is indeed  $< 0.5^\circ$  (ref. 1). We assume here that the photometric phenomenon is also due to localised scattering and shadowing on the rough surfaces of macroscopic ring bodies, rather than to mutual effects between such bodies.

## Conclusion

In other astronomical contexts, for example, in B-emission stars, the presence of a disk-like structure is known or presumed to produce an optical polarisation, correlated in direction with the disk plane. In the most striking example of a disk-like structure in our Solar System, Saturn's rings, we have shown that such an effect is not readily visible. Rather, a competing effect, correlated geometrically not with the disk but with the Sun-planet-Earth plane, is dominant under all conditions, even near exact opposition, at which time the disk-like component should be most readily distinguishable. At least two mechanisms have



been cited for the production of a disk-like polarisation: a monolayer effect; and the rescattering of sunlight reflected from the body of planet, by the rings. The former would operate only to the extent that the disk approximates a monolayer; but the second mechanism is surely applicable. The observed smallness of the contribution to the polarisation due to either or both of these mechanisms,  $\leq 0.1\%$  in blue light, relative to the polarisation of  $\sim 0.5\%$  caused by direct scattering of sunlight by the rough surfaces of individual ring bodies, has been attributed to the optical properties of the ring bodies. We conclude that the large-angle ( $\sim 90^\circ$ ) scattering polarisation of the ring bodies is quite small,  $\ll 10\%$ . This quantitative feature has evidently precluded the detection of a disk-like polarisation. Polarimetric results from Pioneer 11 will be of great interest, specifically with respect to our prediction that the large-angle polarisation of the rings—not directly measurable from Earth—must be very small.

Some investigators<sup>14,18</sup> have supported the hypothesis that the photometric opposition (brightening) effect is caused by shadowing within the surface microstructure of individual ring bodies. Others<sup>19</sup> have argued that the singular magnitude and sharpness of the effect is due mainly to mutual shadowing among the discrete ring bodies, and the opposition brightening by such a mechanism has been extensively modelled<sup>12,19,20</sup>. As the cusp in the  $p(\alpha)$  curve (Fig. 1) is as narrow as the brightness peak, we can presume the polarimetric and photometric opposition effects to be parts of the same process; thus our results, substantiated by Dollfus' data, indirectly support the first of the two hypotheses for the brightening. If the polarisation were controlled by multiple light paths between the ring bodies, rather than by the local rough-surface texture, a ring plane-correlated polarisation would have been detected.

The polarisation phase curves for the rings are rather unusual when compared with those of other planetary objects and laboratory samples. Only a few published cases are known of laboratory samples exhibiting a strong, flat negative branch combined with a very weak positive polarisation at large angles. This may be due to the lack of laboratory data on high-albedo materials, especially frosts. More work on laboratory frosts would be of interest and should include the difficult polarimetric measurements at extremely small phase angles ( $< 0.5^\circ$ ), so that the narrow 'cusp' can be defined.

Finally, more complex models for the rings may be necessary to account for all of the optical phenomena, including the polarisation behaviour. For example, a two-component model involving a haze layer is being considered in connection with the IR emission (R. E. Murphy, personal communication).

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*Note added in proof:* Larry Pleskot (private communication) finds that the sharp opposition effect of Saturn's rings can be approximated in the laboratory with a fine-grained winter frost covering a macroscopically rough substrate. Indeed, one characteristic of high-albedo surfaces seems to be such an opposition effect. He concludes, as we do, that the opposition effect of Saturn's rings cannot be modelled solely on the basis of mutual shadowing of discrete ring bodies, but must include the effects of scattering on the surface of individual bodies.

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## U-Pb, Sm-Nd and Rb-Sr systematics of mid-ocean ridge basalt glasses

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*The measurement of Pb, Nd and Sr isotopes in basalt glasses from mid-ocean ridges reveals correlations in isotope parameters which have important implications for the differentiation history of the mantle.*

THE isotopic compositions of Pb, Nd and Sr in oceanic basalts reflect the fractionation histories of U/Pb, Sm/Nd and Rb/Sr in their mantle sources, and therefore may throw light on several important problems including the differentiation of mantle material to produce continental crust, the recycling of continental material and the degree of convective mixing in the mantle. As mid-ocean ridge basalts comprise nearly 80% of the Earth's

volcanic products they should convey a good impression of the isotopic characteristics of the sub-oceanic mantle. However, ocean island basalts because of their accessibility and state of preservation have attracted much more attention from isotope geochemists despite their comparatively small volume.

Early investigations of the Rb-Sr systematics of oceanic basalts established that mid-ocean ridge basalts often possess Rb/Sr ratios that are too low to generate their measured  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios in  $\sim 4.5$  Gyr from a chondritic initial  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio and further that significant differences exist in  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios from one mid-ocean ridge location to another (see refs 1, 2). The first reliable Pb-isotope compositions obtained from mid-ocean ridge basalts by Tatsumoto<sup>3</sup> showed that the evolution of their source had not involved a large fractionation in U/Pb ratio relative to the assumed whole Earth value. The available data base for Sr- and Pb-isotopes in mid-ocean ridge basalts has recently increased substantially<sup>4-10</sup>, but only rarely

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Table 1 Isotopic analyses of basalt glasses

| Sample  | Long.     | Ref.            | $^{206}\text{Pb}/^{204}\text{Pb}$ | $^{207}\text{Pb}/^{204}\text{Pb}$ | $^{208}\text{Pb}/^{204}\text{Pb}$ | $\text{U}^{238}/\text{Pb}^{204}$ | $^{87}\text{Sr}/^{86}\text{Sr}$ | $^{87}\text{Rb}/^{86}\text{Sr}$ | $^{143}\text{Nd}/^{144}\text{Nd}$ | $^{147}\text{Sm}/^{144}\text{Nd}$ | Sm*  | Nd*   | Rb*   | Sr*  | U*    | Pb*   |
|---------|-----------|-----------------|-----------------------------------|-----------------------------------|-----------------------------------|----------------------------------|---------------------------------|---------------------------------|-----------------------------------|-----------------------------------|------|-------|-------|------|-------|-------|
| A37.18N | 35°12' W  | 3               | 19.20<br>±.03                     | 15.59<br>±.03                     | 38.61<br>±.10                     | 6.2                              | 0.70321<br>±.5                  | 0.105                           | 0.51309<br>±.3                    | 0.226                             | 2.46 | 6.55  | 2.27  | 62.5 | 0.060 | 0.625 |
| A22.59N | 43°31' W  | 4               | 18.20<br>±.02                     | 15.46<br>±.03                     | 37.56<br>±.05                     | 9.64                             | 0.70253<br>±.5                  | 0.028                           | 0.51314<br>±.3                    | 0.209                             | 3.42 | 9.38  | 1.88  | 194  | 0.063 | 0.408 |
| A45.15N | 28°02' W  | 2               | 19.28<br>±.02                     | 15.54<br>±.02                     | 38.83<br>±.05                     | 37.9                             | 0.70314<br>±.4                  | 0.126                           | 0.51309<br>±.3                    | 0.183                             | 2.79 | 9.15  | 4.58  | 105  | 0.195 | 0.332 |
| A11.16N | 43°39' W  | 5               | 18.34<br>±.02                     | 15.47<br>±.01                     | 37.68<br>±.03                     | 4.81                             | 0.70249<br>±.4                  | 0.015                           | 0.51312<br>±.3                    | 0.208                             | 4.13 | 11.92 | 0.66  | 123  | 0.043 | 0.559 |
| AO5.47S | 11°25' W  | 6 <sup>  </sup> | 17.84<br>±.02                     | 15.47<br>±.02                     | 37.33<br>±.05                     | 8.0                              | 0.70230<br>±.4                  | 0.032                           | 0.51329<br>±.2                    | 0.208                             | 4.04 | 11.70 | 1.48  | 133  | 0.118 | 0.91  |
| A59.46N | 29°48' W  | 1               | 18.31<br>±.03                     | 15.53<br>.03                      | 37.90<br>±.07                     | 12.4                             | 0.70279<br>±.4                  | 0.032                           | 0.51323<br>±.3                    | 0.239                             | 2.42 | 6.08  | 0.77  | 70.0 | 0.041 | 0.211 |
| P00.42N | 85°30' W  | 8               | 18.56<br>±.02                     | 15.49<br>±.02                     | 38.07<br>±.04                     | 22.9                             | 0.70275<br>±.5                  | 0.132                           | 0.51308<br>±.3                    | 0.187                             | 7.85 | 25.2  | 3.35  | 73.0 | 0.231 | 0.636 |
| P44.40N | 130°30' W | 7               | 18.43<br>±.03                     | 15.44<br>±.03                     | 37.64<br>±.06                     | 17.6                             | 0.70253<br>±.4                  | 0.045                           | 0.51313<br>±.3                    | 0.200                             | 4.86 | 14.6  | 1.79  | 114  | 0.137 | 0.502 |
| P08.16S | 108°50' W | 9               | 18.46<br>±.02                     | 15.48<br>±.02                     | 37.97<br>±.04                     | 5.01                             | 0.70265<br>±.5                  | 0.007                           | 0.51302<br>±.2                    | 0.185                             | 3.13 | 10.19 | 0.392 | 156  | 0.039 | 0.491 |
| I50.25S | 131°00' E | 11              | 18.75<br>±.02                     | 15.49<br>±.02                     | 38.19<br>±.05                     | 12.5                             | 0.70261<br>±.4                  | 0.034                           | 0.51305<br>±.2                    | 0.180                             | 4.77 | 15.9  | 2.11  | 180  | 0.134 | 0.681 |
| I14.45N | 54°48' E  | 10              | 18.43<br>±.03                     | 15.43<br>±.03                     | 38.01<br>±.06                     | 6.3                              | 0.70278<br>±.5                  | 0.013                           | 0.51302<br>±.2                    | 0.192                             | 1.84 | 5.77  | 0.35  | 78.4 | 0.023 | 0.230 |

Source of samples: (1) Reykjanes Ridge P601; (2) 45°N 56 (from F. Aumento); (3) DSDP 335-5-2, 5; (4) DSDP 396-18-3, 58; (5) Vema Fracture Zone 111327 (ref. 25); (6) AD3-3 (ref. 40); (7) Juan de Fuca Ridge 11240/60 (ref. 39); (8) Galapagos Ridge P115 (from W. G. Melson) described in ref. 41; (9) East Pacific Rise P6707-40 (from E. Bonatti); (10) Gulf of Aden 24B (from E. Bonatti); (11) Vema 33 Dredge (Lamont-Doherty Geological Observatory).

\* All concentration data in  $\mu\text{g per g}$ .

†  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios expressed relative to  $^{87}\text{Sr}/^{86}\text{Sr} = 0.70800$  for Eimer and Amend  $\text{SrCO}_3$ . Measured value for this standard is 0.70806.

‡ Normalised to  $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$ .

§ Data from NBS-981 indicates a fractionation of  $-0.6 \pm 0.2\%$ .  $\text{AMU}^{-1}$ . No correction made.

|| U and Pb concentration data from ref. 22.

have Pb- and Sr-isotopes been measured on the same samples. In the past few years Nd-isotope compositions have been obtained on mid-ocean ridge and oceanic island basalts<sup>11-14</sup> and in those instances where Sr-isotope compositions have been reported for the same samples there is a clear negative correlation between  $^{143}\text{Nd}/^{144}\text{Nd}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios. This correlation attests to a strong coherence in the fractionation of Rb/Sr and Sm/Nd during the evolution of the basalt sources. A similar comparison of the behaviour of U/Pb with Rb/Sr and Sm/Nd in oceanic basalt sources is more difficult, but the compilation of Pb- and Sr-isotope data made by Sun and Hanson<sup>15</sup> suggests that overall no such simple coherence exists. Further evaluation of these relationships has been hampered by the fact that Pb-, Nd- and Sr-isotope compositions have not been obtained on the same samples. An additional complication is that both Rb/Sr and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios of ocean floor basalts may be modified by alteration processes operating on the sea floor. However, it has been shown that the glassy rims of pillow basalts generally provide the most reliable estimates of Rb/Sr and  $^{87}\text{Sr}/^{86}\text{Sr}$  (ref. 2) and that the U-abundances of submarine basalts may be modified by interaction with seawater<sup>16,17</sup>. The reliability of many of the published Rb-Sr and U-Pb data on submarine crystalline basalts are thus very difficult to evaluate. The present study aimed to improve the existing situation by measuring  $^{208}\text{Pb}/^{204}\text{Pb}$ ,  $^{207}\text{Pb}/^{204}\text{Pb}$ ,  $^{206}\text{Pb}/^{204}\text{Pb}$ ,  $^{143}\text{Nd}/^{144}\text{Nd}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios together with the abundances of U, Pb, Sm, Nd, Rb and Sr all on the same samples of mid-ocean ridge basalt glasses from the Atlantic, Indian and Pacific oceans.

## Analytical techniques

To minimise the possibility of contamination, glass rather than crystalline material was carefully handpicked from drilled or dredged basalt samples. Typically an aliquot of glass between 50 and 150 mg was dissolved and Pb separated electrochemically using a miniaturised version of the technique described by Arden and Gale<sup>18</sup>. Sr and Nd were subsequently isolated from the samples using cation and anion exchange techniques respectively<sup>12</sup>. U, Pb, Sm, Nd, Rb and Sr abundances were determined on a second aliquot of glass by mass spectrometric isotope dilution analysis. The purification techniques used were the same as the above with the addition of a reverse phase

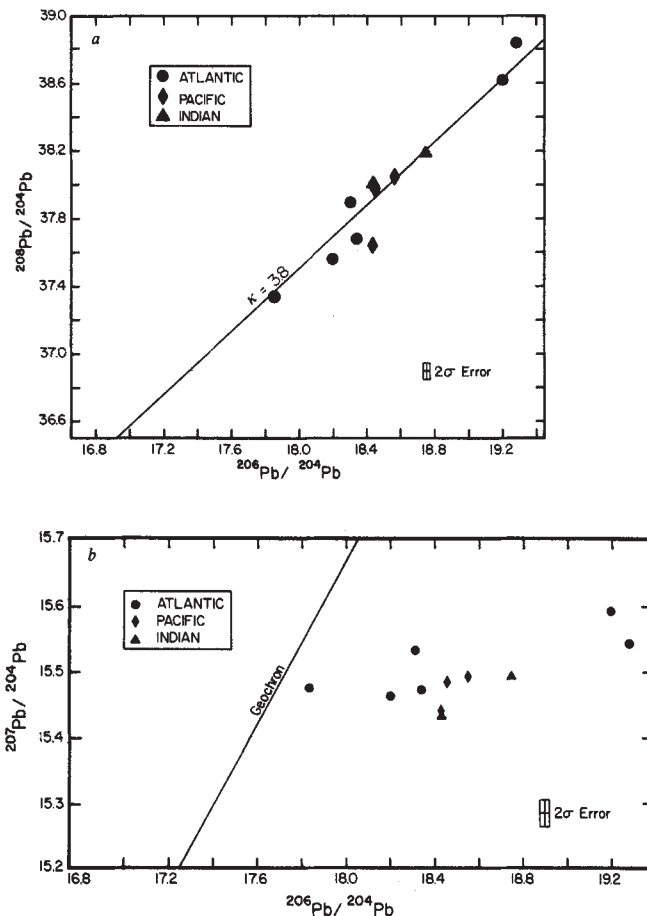


Fig. 1 a, Plot of  $^{208}\text{Pb}/^{204}\text{Pb}$  versus  $^{206}\text{Pb}/^{204}\text{Pb}$  for basaltic glass samples from the Atlantic, Indian and Pacific oceans. The line drawn is for a value of  $\kappa$  ( $^{232}\text{Th}/^{238}\text{U}$ ) = 3.8. The errors indicated are average errors (Table 1). b, Plot of  $^{207}\text{Pb}/^{204}\text{Pb}$  versus  $^{206}\text{Pb}/^{204}\text{Pb}$  for basaltic glass samples from the Atlantic, Indian and Pacific oceans. The geochron has been constructed for 4.55 Gyr assuming initial Pb isotope compositions of Canyon Diablo<sup>42</sup>. The errors indicated are average errors for the analyses (see Table 1).



chromatographic procedure for the separation of U (ref. 19). The mass spectrometric techniques for the isotopic analysis of Pb, Rb, Sr, Sm and Nd have been described previously<sup>12,20</sup> and U was isotopically analysed using a triple-filament technique and ion-counting with a Daly detector. Procedural blanks are as follows: U = 0.01 ng; Pb = 0.5 ng; Sm < 0.05 ng; Nd < 0.05 ng; Rb = 0.05 ng; Sr = 0.1 ng.

Major element analyses of the glasses were obtained using an electron probe with energy dispersive analysis at the Department of Mineralogy and Petrology, Cambridge.

## Results

The  $^{208}\text{Pb}/^{204}\text{Pb}$ ,  $^{207}\text{Pb}/^{204}\text{Pb}$ ,  $^{206}\text{Pb}/^{204}\text{Pb}$ ,  $^{143}\text{Nd}/^{144}\text{Nd}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios together with U, Pb, Sm, Nd, Rb and Sr concentrations for 11 mid-ocean ridge basalt glass samples are given in Table 1 and their major element compositions are listed in Table 2. The sample numbering system in Tables 1 and 2 is similar to that used by Melson *et al.*<sup>21</sup> where the letter prefix (A, I or P) denotes the ocean concerned and the following sequence of numbers and letter denote the latitude. Although the data in Table 1 are not fully representative of the Earth's mid-ocean ridge basalts they are adequate to demonstrate the nature of the isotopic variations that exist.

The Pb-isotope compositions for all of the samples are compared on plots of  $^{208}\text{Pb}/^{204}\text{Pb}$  and  $^{207}\text{Pb}/^{204}\text{Pb}$  versus  $^{206}\text{Pb}/^{204}\text{Pb}$  in Fig. 1a,b. The  $^{207}\text{Pb}/^{204}\text{Pb}$  and  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios of the samples all plot to the right of the geochron (4.55 Gyr isochron) and exhibit a positive correlation similar to that noted previously for mid-ocean ridge basalts<sup>7,22</sup>. The range of Pb-isotope compositions in the Atlantic samples is greater than that for either the Indian or Pacific oceans, but this may only reflect the limited size of the sample set.

$^{143}\text{Nd}/^{144}\text{Nd}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios are compared with the trend previously defined by mid-ocean ridge and ocean island basalts in Fig. 2. The samples exhibit a considerable range in both  $^{87}\text{Sr}/^{86}\text{Sr}$  and  $^{143}\text{Nd}/^{144}\text{Nd}$  ratios, and conform closely to the previously documented trend for oceanic volcanics. The highest  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios are found in basalt glasses from 37°N and 45°N on the Mid-Atlantic Ridge (A37.18N and A45.15N) close to the Azores platform (refs 10, 23). Conversely the sample with the lowest  $^{87}\text{Sr}/^{86}\text{Sr}$  and highest  $^{143}\text{Nd}/^{144}\text{Nd}$  ratio is from the Mid-Atlantic Ridge at 6°S (A05.47S).

The most important new observation to be made about these data is probably the association of the least radiogenic Pb isotope and Sr isotope compositions and vice versa (Fig. 3). Overall there is a good positive correlation between the  $^{87}\text{Sr}/^{86}\text{Sr}$  and  $^{206}\text{Pb}/^{204}\text{Pb}$  which indicates a strong coherence between U/Pb and Rb/Sr during the evolution of the mid-ocean ridge basalt source region. Note that the Pb- and Sr-isotope data reported for Icelandic and Reykjanes Ridge basalts<sup>8,24</sup> are consistent with the correlation shown in Fig. 3, but in general, data reported from other oceanic islands do not show such a consistent correlation (see compilation in ref. 15).

The abundances of U, Pb, Rb, Sr, Sm and Nd in the basalt glasses show considerable variation (Table 1), for example, Rb/Sr ratios vary by more than an order of magnitude from

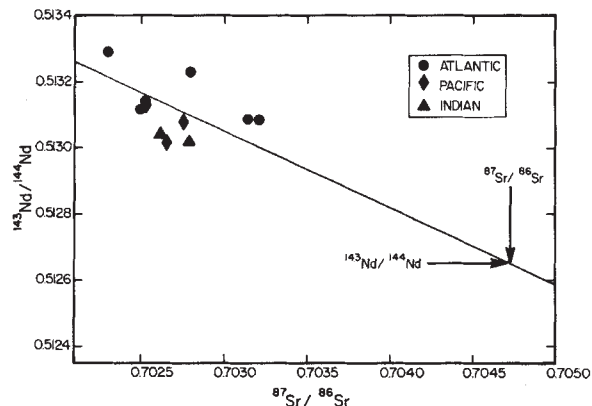


Fig. 2 Comparison of  $^{143}\text{Nd}/^{144}\text{Nd}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios of basalt glasses from the Atlantic, Indian and Pacific oceans with the trend defined previously for oceanic volcanics<sup>11-13</sup>. The arrows indicate the estimated  $^{143}\text{Nd}/^{144}\text{Nd}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios of the bulk Earth<sup>12,13</sup>.

0.003 to 0.044 and U/Pb ratios range from 0.077 to 0.587. With the exception of sample A37.18N the Rb/Sr and U/Pb ratios are positively correlated (Fig. 4). A second and apparently pure 5-mg aliquot of A37.18N has been analysed and found to have Rb/Sr = 0.065, a factor of two higher than the first sample. The reason for the highly variable Rb abundance in this glass is not understood. Correlations between  $^{87}\text{Rb}/^{86}\text{Sr}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  and  $^{238}\text{U}/^{204}\text{Pb}$  and  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios respectively are poor as seen in a plot of  $^{238}\text{U}/^{204}\text{Pb}$  versus  $^{206}\text{Pb}/^{204}\text{Pb}$  in Fig. 5 and do not provide any evidence for discrete mantle fractionation events.

The major element analyses of the samples (Table 2) are typical of those for mid-ocean ridge tholeiites (see, for example, ref. 25) with  $\text{SiO}_2$  contents ranging from 49.12 to 52.47 wt %. None of the major element abundances or ratios such as  $\text{MgO}/(\text{MgO} + \text{FeO})$  show any clear correlation with trace element abundances, or abundance ratios such as Rb/Sr or U/Pb.

## Discussion

The  $^{143}\text{Nd}/^{144}\text{Nd}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios of the glasses are respectively greater than and less than the model bulk Earth values for these ratios and are consistent with the negative correlation identified previously for oceanic basalts<sup>10-12</sup>. It has been suggested that a unidirectional transport model involving the extraction of silicate melt from the mantle to leave a residuum with lower Rb/Sr and higher Sm/Nd ratio than the original would produce with time the Nd- and Sr-isotope characteristics of mid-ocean ridge basalts<sup>12,26-28</sup>. The general correlation between  $^{206}\text{Pb}/^{204}\text{Pb}$  and  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios in mid-ocean ridge basalts suggests that there was a coherence between U/Pb and Rb/Sr during the evolution of the basalt source region. Is this correlation consistent with the simple unidirectional transport models that have been used to accommodate the Nd and Sr data? An assessment of this possibility is made difficult by uncertainties in the values of U and Pb distribution coefficients

Table 2 Major element analyses of MORB glasses

|                         | A59.46N | A45.15N | A37.18N | A22.59N | A11.16N | A05.47S | P44.40N | P00.42N | P08.16S | I14.45N | I50.25S |
|-------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| $\text{SiO}_2$          | 52.47   | 52.20   | 50.90   | 49.68   | 50.06   | 49.48   | 51.54   | 49.85   | 49.12   | 51.32   | 50.90   |
| $\text{TiO}_2$          | 1.10    | 0.98    | 0.98    | 1.18    | 1.45    | 1.39    | 1.88    | 3.26    | 1.37    | 0.87    | 2.12    |
| $\text{Al}_2\text{O}_3$ | 13.54   | 14.75   | 15.06   | 15.25   | 15.76   | 16.72   | 13.47   | 11.69   | 16.82   | 15.30   | 15.25   |
| $\text{FeO}$            | 12.03   | 9.47    | 9.02    | 8.43    | 9.99    | 8.62    | 11.63   | 16.72   | 9.00    | 9.44    | 10.02   |
| $\text{MnO}$            | 0.15    | —       | —       | —       | 0.17    | 0.19    | 0.10    | 0.09    | —       | 0.08    | 0.16    |
| $\text{MgO}$            | 6.83    | 9.13    | 7.75    | 7.97    | 7.87    | 8.20    | 6.73    | 4.86    | 8.58    | 8.57    | 8.02    |
| $\text{CaO}$            | 11.83   | 11.31   | 11.51   | 11.01   | 11.13   | 11.14   | 11.00   | 9.25    | 10.76   | 12.22   | 10.27   |
| $\text{Na}_2\text{O}$   | 2.21    | 2.04    | 2.39    | 2.47    | 2.79    | 2.66    | 2.67    | 2.49    | 3.24    | 2.35    | 2.89    |
| $\text{K}_2\text{O}$    | —       | 0.18    | —       | 0.06    | 0.12    | 0.24    | 0.06    | 0.16    | —       | —       | 0.22    |
|                         | 100.16  | 100.06  | 97.61   | 96.05   | 99.34   | 98.64   | 99.08   | 98.37   | 98.89   | 100.15  | 99.85   |

Dash indicates element below level of detectability with counting times used. A05.47S from Engel and Engel (ref. 40). A11.16N from Melson and Thompson (ref. 25).

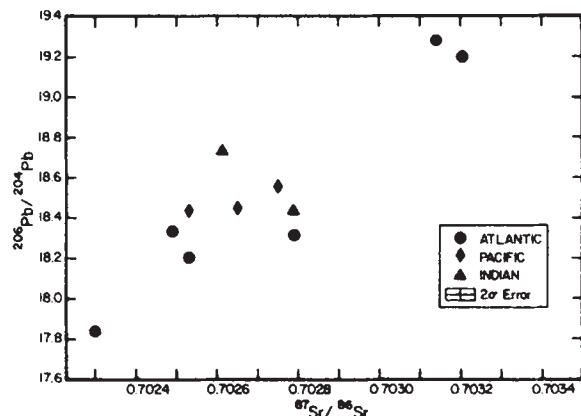


Fig. 3 Plot of  $^{206}\text{Pb}/^{204}\text{Pb}$  versus  $^{87}\text{Sr}/^{86}\text{Sr}$  for basaltic glasses from Atlantic, Indian and Pacific oceans. Average error is indicated.

in silicate systems, by the role of minor U- and Pb-bearing phases in the source region, and the uncertainty of the bulk Earth lead isotope composition. Whereas some ocean island basalts have  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios higher than any reasonable estimate of the bulk Earth Pb-isotope composition<sup>29,30</sup>, the relationship of the Pb-isotope compositions of mid-ocean ridge basalts to the bulk Earth is less certain. If the bulk Earth Pb-isotope composition lies on the geochron (plotted for an assumed of 4.55 Gyr) in Fig. 1b, as is generally assumed, then all of the ocean ridge basalts under discussion here have  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios higher than the bulk Earth value. If the previous interpretations of the Nd and Sr-isotope correlation in oceanic basalts are applied to the above Pb- and Sr-isotope data, then those samples with highest  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios are considered to have been derived from less depleted (in a time-integrated sense) mantle than samples with lower  $^{87}\text{Sr}/^{86}\text{Sr}$ . Extrapolation of the  $^{206}\text{Pb}/^{204}\text{Pb}$  versus  $^{87}\text{Sr}/^{86}\text{Sr}$  correlation to a model  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio of the bulk Earth ( $\sim 0.7050$ ) would suggest that the  $^{206}\text{Pb}/^{204}\text{Pb}$  ratio of the bulk Earth is  $\sim 21.3$ , a much greater value than other estimates of the bulk Earth Pb-isotope compositions. Furthermore if a unidirectional transport process effected a decrease in Rb/Sr and a concomitant increase in U/Pb in the residual mantle, then the lowest  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios would be associated with the highest  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios—the opposite to what is observed! Clearly a more complex model of Earth evolution than a simple unidirectional transport model, is required to accommodate the Pb-, Nd- and Sr-isotope data.

At this stage several more complex models should be considered. For example, it has been suggested<sup>31,32</sup>, that Pb may have been partitioned into sulphide phases preferentially to U and subsequently located in the core during Earth differentiation. If, for example, such a process increased the U/Pb ratio in some part of the mantle, but not the Rb/Sr ratio, then at present it would have a  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio of 0.705 (the bulk Earth value) and

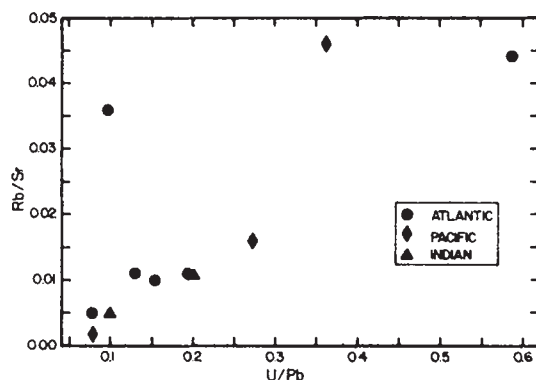


Fig. 4 Plot of U/Pb and Rb/Sr in basaltic glasses from the Atlantic, Indian and Pacific oceans.

a  $^{206}\text{Pb}/^{204}\text{Pb}$  ratio in excess of the bulk Earth value. To produce the correlation in Fig. 3 this source or magmas derived from it would have to mix with those from another mantle source that had suffered a reduction in Rb/Sr ratio but comparatively little change in U/Pb ratio. Alternatively other workers have used models involving bidirectional transport of material between the mantle and the continental crust<sup>33,34</sup> to generate the Pb- and Sr-isotope compositions of oceanic volcanics. Recently Doe and Zartman<sup>35</sup>, O'Nions *et al.*<sup>36</sup> and Evensen *et al.*<sup>37</sup> have pointed out that the continental crust may be considered as two reservoirs as far as Pb-isotopes are concerned. Because U is preferentially concentrated in the upper parts of the continental crust, the upper crust evolves with a relatively more radiogenic Pb-isotope composition than the lower part. Any preferential recycling of upper crust into the mantle would result in a mean Pb-isotope composition of the mantle more radiogenic than the bulk Earth and could account for the observed range of Pb-isotope composition found in oceanic basalts. Whether or not all the features of Pb-, Nd- and Sr-isotope chemistry of mid-ocean ridge basalt glasses can be reproduced by such models of continent recycling must await the results of further computations. Nevertheless, adopting such models would implicitly favour a continuous rather than episodic evolution of Pb-isotopes in the mantle.

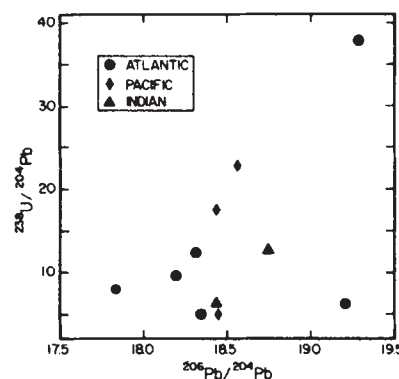


Fig. 5 Plot of  $^{238}\text{U}/^{204}\text{Pb}$  versus  $^{206}\text{Pb}/^{204}\text{Pb}$  for basaltic glasses from the Atlantic, Indian and Pacific oceans.

The comparatively small variations in Pb-, Nd- and Sr-isotope compositions in the glasses require that the time-integrated (over the duration of Earth history) Rb/Sr, U/Pb and Sm/Nd ratios in the various source regions must have been quite uniform. This is in marked contrast to the order of magnitude variation of Rb/Sr and U/Pb in the glasses. Such large variations could not be generated from a uniform source composition if the partial melting which produced the basalts followed a simple equilibrium melting model (for example see ref. 38) and the primary magmas produced underwent only small amounts of subsequent crystallisation differentiation. Thus it must be inferred either that the processes of basalt magma generation and evolution of mid-ocean ridges can effect much greater fractionation of Rb/Sr, U/Pb and Sm/Nd than is often appreciated or alternatively some mantle process effects large, but transient fractionation before the melting event. The petrogenetic utility of trace element abundances in oceanic basalts is severely limited until some resolution of these alternatives can be made.

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# Tholeiitic basalt–rhyolite magmatism and massive sulphide deposits at Matagami, Quebec

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*Noranda-type massive sulphide deposits in some Archean greenstone belts of the Canadian Shield occur in tholeiitic volcanic sequences. The tholeiitic rocks belong to basalt–rhyolite bimodal suites with low  $Al_2O_3$ , high Na/K ratios, high iron contents, and well-defined iron enrichment trends.*

In Archean greenstone belts of the Canadian Shield Fe–Zn–Cu massive sulphide deposits of the Noranda-type are invariably described as occurring in calc-alkaline volcanic rocks<sup>1–4</sup>. Within each belt the volcanic succession typically consists of a laterally extensive sequence of tholeiitic basalt forming the lower two-thirds of the stratigraphy (Fig. 1a), overlain by volcanic rocks of calc-alkaline attribution (basalt, andesite, dacite and rhyolite) that form thick accumulations at discrete volcanic centres<sup>1,5–7</sup>. The accompanying massive sulphide deposits, ranging up to  $2 \times 10^8$  tonnes in size (~5% Zn, 2% Cu), are composed mainly of pyrite, pyrrhotite, sphalerite and chalcophyrite, and occur as stratiform lenses clustered within the 'calc-alkaline' centres, most commonly on the flanks of rhyolite domes or other felsic accumulations. The sulphides were deposited as chemical sediments on the ancient seafloor<sup>8</sup>, around or downslope from exhalation centres (now mineralised alteration pipes) that emitted hot metalliferous brines akin to those now found at the bottom of the Red Sea<sup>9</sup>. The massive sulphides thus form part of the original stratigraphy, and their apparent association with calc-alkaline rocks has historically made the latter an essential criterion for defining mineral exploration targets. However, in one of these large Archean volcanic centres, at Matagami, Quebec, this relationship does not hold; the massive sulphide deposits, while originally thought to lie within calc-alkaline rocks<sup>10</sup>, are now shown to occur within a bimodal basalt–rhyolite suite of tholeiitic affinity<sup>11,12</sup>. Their apparent 'calc-alkaline' affinity is due to the widespread hydrothermal alteration that accompanied sub-seafloor geothermal activity, as will be dis-

cussed elsewhere. In this article we present evidence of the tholeiitic nature of these rocks, discuss their relationship to the ore deposits, and suggest that other Noranda-type massive sulphide deposits may occur in tholeiitic volcanic centres.

## Geology of the Matagami district

The pertinent stratigraphy at Matagami consists of 80% basalt and 20% rhyolite (Fig. 1b,c). It contains over 18 mostly Zn-rich stratiform massive sulphide lenses, all closely associated with rhyolites. These rocks and ores have been intruded by the Bell River Igneous Complex and many of its subsidiary dykes and sills, then metamorphosed to the greenschist facies, and folded into a westward plunging anticline<sup>13</sup> (Fig. 2). The sulphide

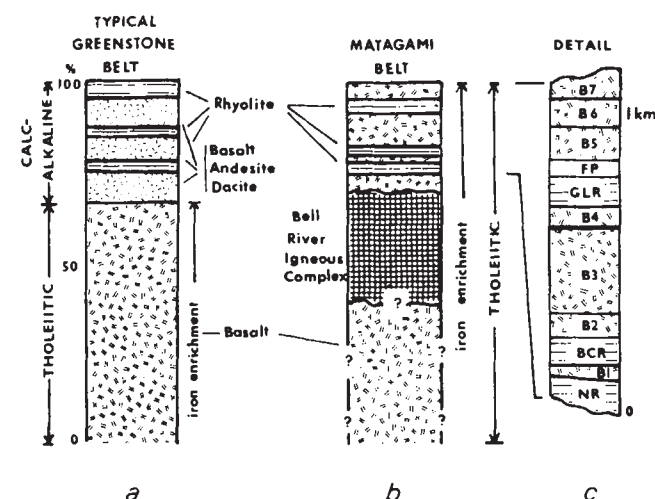


Fig. 1 Comparison of the stratigraphy in a typical Archean greenstone belt: a, to that at Matagami; b, with detail for Matagami in c. B1–B7 are basalts; NR, Norita rhyolite; BCR, Bell Channel rhyolite; GLR, Garon Lake rhyolite; FP, feldspar porphyry (andesite).

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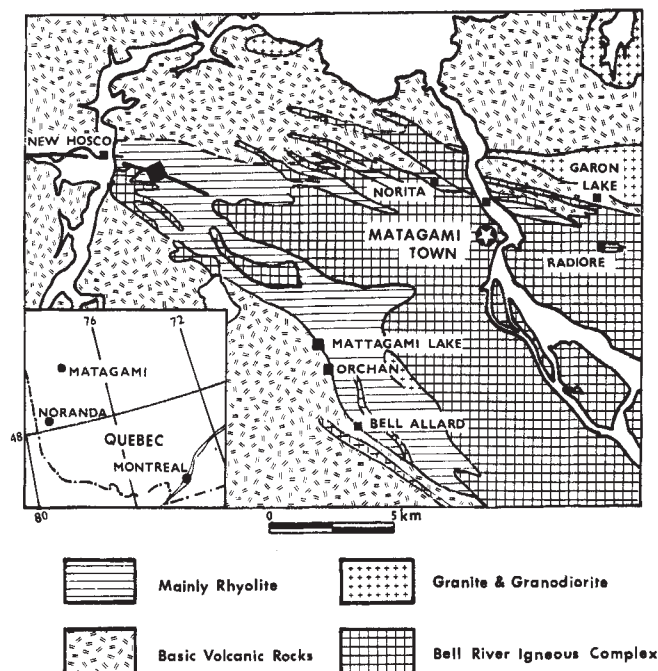


Fig. 2 Simplified geology of the Matagami district (after Sharpe<sup>13</sup>).

deposits, ranging up to 25 Mtonnes in size, occur within a 1 km thick stratigraphic section, and they now form two linear mineralised belts, one on either side of the anticline. On the south limb the deposits (Bell Allard, Orchan, Matagami Lake, New Hosco) are all located at the base of the 'Key Tuffite'<sup>14,15</sup>, a cherty tuffaceous unit that lies on top of a massive rhyolite, whereas those on the north limb are associated with rhyolites at several stratigraphic horizons: Norita rhyolite (Norita mine, Bell Channel No. 1), Bell Channel rhyolite (Bell Channel No. 4, Radiore), and Garon Lake rhyolite (Garon Lake mine)<sup>11,12</sup>.

## Stratigraphy

The volcanic rocks on the north limb of the anticline have been mapped and sampled in detail, and each unit has been analysed for major and several trace elements (Fig. 4). The stratigraphic sequence is composed of seven pillowed basalt units, a pillowed andesite and three rhyolite flows (Fig. 1c). The basalts are mostly aphyric or weakly plagioclase-phyric, but one unit, B2, is coarse grained throughout and contains abundant phenocrysts of plagioclase and pyroxene (now uraltised) in about equal proportions. The three rhyolite units are spherulitic flows which form domal accumulations up to 150 m thick, with associated pyroclastic material. All three units contain phenocrysts of quartz (inverted  $\beta$ -quartz) and sodic plagioclase, and rare granophyre xenoliths (Fig. 3a). The Bell Channel rhyolite also

contains microphenocrysts of tridymite (inverted) (Fig. 3b)<sup>12</sup>.

These volcanic rocks are intruded by concordant gabbro sills ranging from 50 to 250 m thick which grade laterally into underlying feeder dykes connected to the Bell River Igneous Complex. Contact features bordering these minor intrusions indicate they were emplaced contemporaneously with the extrusive rocks, and some indeed may represent lateral feeders to overlying flows<sup>11</sup>.

## Petrology and primary geochemical trends

All the volcanic rocks in the district have undergone a weak pervasive hydration, but alteration was more intense in the vicinity of the sulphide deposits; there, the rhyolites are chloritised and the underlying basalts are strongly spilitised and silicified. However, the stratigraphic sequence was extensively sampled for more than 5 km along strike, and the primary geochemical features of each rock unit were established (Fig. 4) using samples in which the primary mineral fabric was well preserved.

**Basalt:** the seven basalt units consist of plagioclase (sauritised), pyroxene (uralitised), and minor opaque oxide set in devitrified glass that is either chloritic (B2–B4) or quartzofeldspathic (B5–B7). They are all quartz normative and have low  $Al_2O_3$ , high Na/K ratios, and high iron contents. Plots of the oxides against differentiation index<sup>16</sup> (Fig. 4) show minor fractionation of the units, but they all cluster at the basaltic end of the diagram, well separated from plots of the rhyolites. On an AFM diagram (Fig. 5a) the basalts (B2–B5) follow a progressive iron enrichment trend within the tholeiitic field, and this is re-emphasised on an MgO versus  $FeO^*$  diagram (Fig. 5b; plots of  $Fe/(Fe+Mg)$  versus Si (refs 17, 18),  $FeO^*$  or  $SiO_2$  versus  $FeO^*/MgO$  (ref. 19) similarly emphasise this relationship). Rocks of unit B5, which show maximum iron enrichment, first crystallised plagioclase and pyroxene, followed by magnetite, sodic feldspar, quartz and albite—the latter two minerals forming a spherulitic mesostasis to the other grains (Fig. 3c). This paragenesis provides strong petrographic support for a tholeiitic trend, and shows the transition from iron enrichment to alkali enrichment during the crystallisation of individual pillows.

**Rhyolite:** the three rhyolite units have high  $SiO_2$  contents, but low  $Al_2O_3$ , high Na/K ratios, and high iron contents, like the basalts (Fig. 4). They plot at the alkali end of the tholeiitic field on an AFM diagram (Fig. 5a), but have normative plagioclase compositions around  $An_{20}(An_{18.5-23})$  and are thus 'dacites' according to most rock classifications<sup>20,21</sup>. There is corroborating petrographic evidence that the plagioclase phenocrysts originally had this composition. All three units contain quartz and plagioclase phenocrysts and micrographic intergrowths (Fig. 3a), indicating they were crystallising on a cotectic in the Q–Or–Ab–An system before extrusion. However, in at least one unit (BCR),  $\beta$ -quartz was succeeded by tridymite after extrusion (Fig. 3b), yielding a minimum magma temperature of 867 °C (ref. 22) but probably as high as 935 °C (ref. 12). These temperatures are well above the liquids of melts crystallising albite, but close to that of melts crystallising plagioclase  $An_{20}$  in

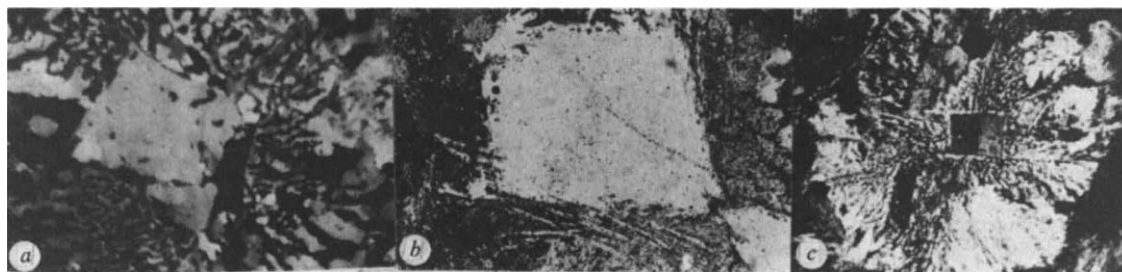


Fig. 3 a, From a granophyre fragment in the Garon Lake rhyolite; central crystal is inverted  $\beta$ -quartz surrounded by a micrographic intergrowth of quartz (white) and albite (grey-black). b, Tridymite phenocryst with reticulate borders, flanked by elongate tridymite microlites, now inverted to quartz (from the Bell Channel rhyolite). c, Spherulitic quartz-albite intergrowth mantling plagioclase and magnetite grains in the mesostasis of B5 basalt.



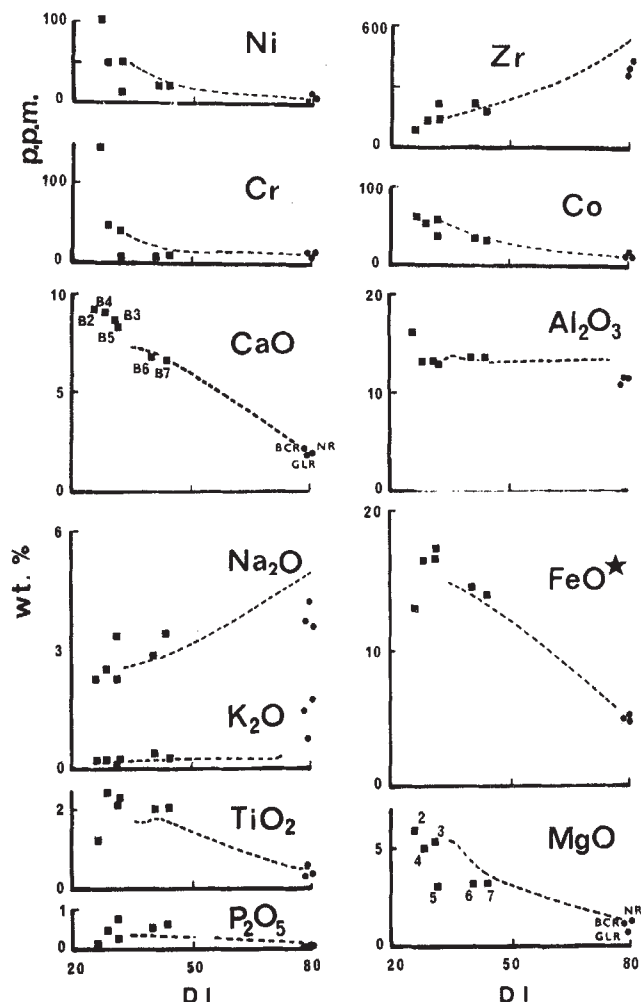


Fig. 4 Major oxides and trace elements<sup>11,12</sup> plotted against differentiation index (DI) show the bimodal distribution of the volcanic suite. ■, basalts; ●, rhyolites. The dashed line is the liquid-line-of-descent for the gabbro-granophyre sill<sup>12</sup> that was intruded contemporaneously with volcanism.

the quinary system Q-Or-Ab-An-H<sub>2</sub>O at 2 kbar pressure<sup>23,24</sup>. As noted above, silic rocks with these high normative An contents are generally classed as dacite, but our three units have SiO<sub>2</sub> contents >10% higher than equivalent dacites in Nockolds' classification<sup>25</sup>. Thus, although these rocks are actually dacites, we will follow the usage of other workers in the Canadian Shield for the time being who call these rocks rhyolite based on their high SiO<sub>2</sub> content<sup>4,27,28</sup> and the present albitic composition of the plagioclase phenocrysts.

### Contemporaneous gabbro sills

The contemporaneous gabbro sills have compositions very similar to the basalts<sup>12,13</sup>, and each sill fractionated along an iron enrichment trend. One highly fractionated gabbro-granophyre sill was studied in detail<sup>12</sup>, and its chemical composition and fractionation trend (Figs 4, 5) place it well within the field of tholeiitic rocks, where it has an iron enrichment trend comparable with that of the well established tholeiitic suite from the Thingmuli Central Volcanic Complex in Iceland<sup>18</sup> (Fig. 5b). The trend in the liquid line-of-descent of this sill (dashed lines, Figs 4, 5) is believed to provide a good approximation of the probable fractionation path of the volcanic rocks at Matagami<sup>12</sup>.

### Interpretation

Using differentiation index as a monitor of progressive fractionation (Fig. 4) the aphyric basalts have chemical compositions essentially identical to successive liquids in the gabbro-grano-

phyre sill (Fig. 4), and follow a comparable but more extreme iron enrichment trend (Fig. 5). Furthermore, each successive flow is more fractionated, indicating that the whole suite was derived from a common parent by progressive fractionation. This is supported by the trace element distributions in the basalts. Cr, Ni and Co have high concentrations in the earliest basalt flows, and decrease rapidly with fractionation, whereas Zr has a low initial value but becomes progressively enriched in the later flows. This is identical to their distribution in the gabbro-granophyre sill (dashed line, Fig. 4), and similar to that of Skaergaard<sup>28</sup>, Thingmuli<sup>29</sup>, and other tholeiitic suites. Preliminary studies on the REE content of the basalts and the sill indicate both have similar distribution patterns (Fig. 6a). These are typical of tholeiites in their low REE content and lack of LREE enrichment, and similar to those of modern oceanic basalts<sup>30</sup> and to that of the Skaergaard liquid after crystallisation of the Middle Zone<sup>31</sup>. Although highly fractionated, they also plot in the field of 'ocean floor' and 'within-plate' tholeiites on a Ti-Zr-Y diagram<sup>32</sup> (Fig. 6b).

The rhyolites and the granophyre of indicated tholeiitic affinity, have similar mineralogy and texture and almost identical composition (Fig. 4). However, both have significantly higher FeO\*, MgO (and CaO), and higher temperature of crystallisation than the Thingmuli rhyolites (Fig. 5b), consistent with their calcic compositions. The granophyre represents a

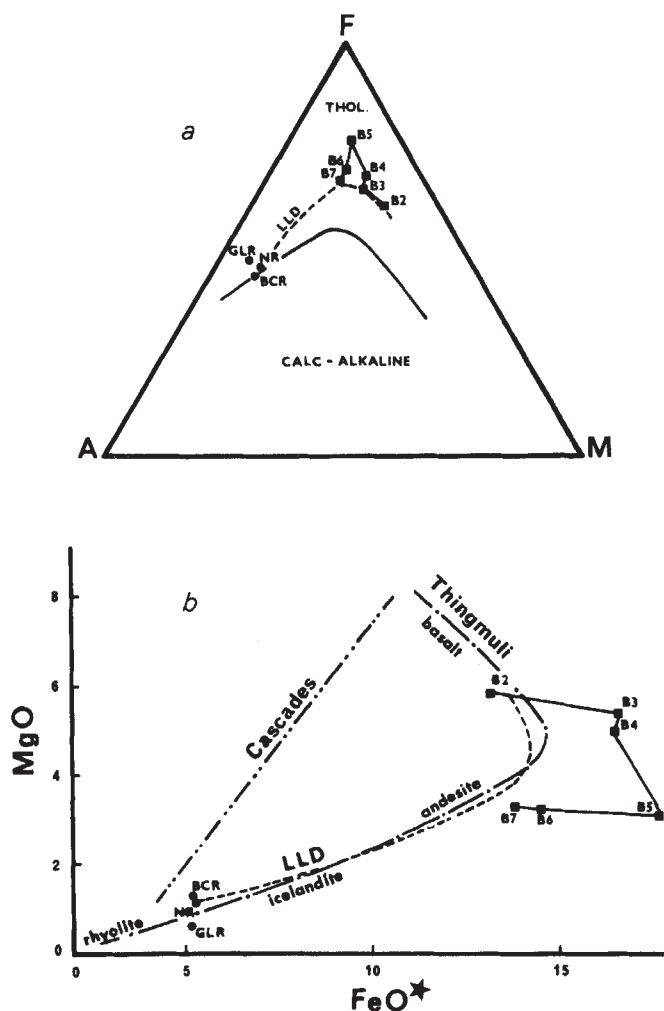


Fig. 5 a, AFM plot (wt%) of the volcanic units and the liquid-line-of-descent (LLD) of the gabbro-granophyre sill (dashed). The dividing line (solid line) for the tholeiitic and calc-alkaline fields is from Irvine and Baragar<sup>20</sup>. b, MgO versus FeO\* (total iron) plot with comparison to Thingmuli (tholeiitic) and Cascades (calc-alkaline).

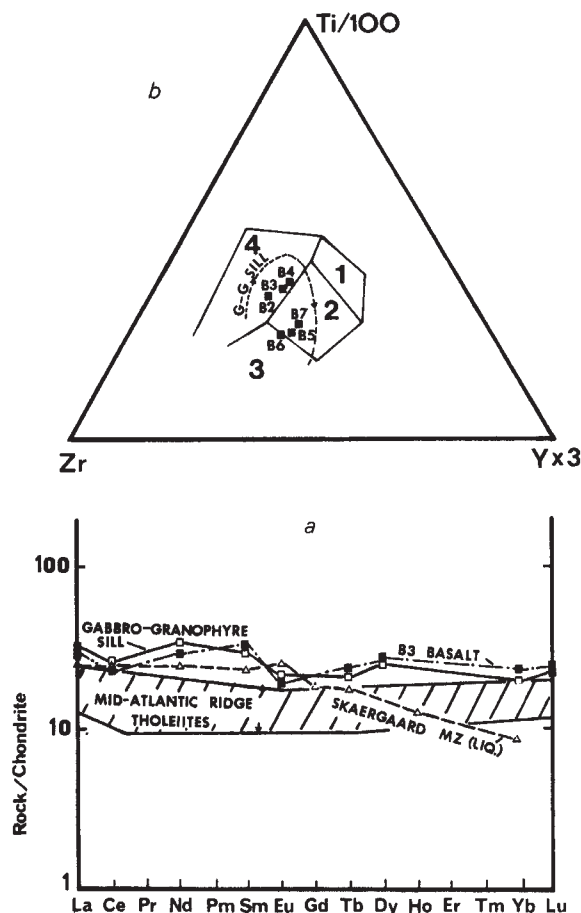


Fig. 6 a, Rare earth element analyses for B3 basalt and the gabbro-granophyre sill, compared to the middle zone (MZ) liquid of the Skaergaard intrusion<sup>31</sup> and the Mid-Atlantic Ridge tholeiites from the FAMOUS expedition<sup>30</sup>. b, Ti-Zr-Y plot<sup>32</sup> of basalts and sill. Fields 1 and 2 are low-K basalts; 2 is ocean floor tholeiite; 3 is calc-alkaline basalt; and 4 is within-plate basalt. Analyses for REE were by neutron activation at X-ray Assay Laboratories, Toronto, Canada, and chondrite-normalised using the values for the Leedy chondrite<sup>46</sup>.

residual melt derived by fractionation of a parental magma very similar in composition to the basalts (dashed line, Fig. 4), leaving but little doubt that it and the rhyolites shared a common origin—as residual melts derived through progressive fractionation of a parental tholeiitic basaltic magma.

Preliminary studies on the underlying Bell River Igneous Complex indicate that its rocks too are very iron-rich<sup>33</sup>, and the layered gabbro-anorthosite series is not disrupted by gabbro dykes and sills that transect the overlying rocks. We interpret these field and chemical relations to indicate a comagmatic source, with the Complex emplaced high into the volcanic pile (probably within 1 km of the surface), and itself acting as a holding chamber while supplying differentiated magma to overlying flows and sills.

Matagami lies at the western end of a linear belt of mixed tholeiitic volcanic rocks and layered gabbroic and anorthositic plutons which includes the Chibougamau mining district 250 km to the east<sup>34</sup>. In geologically younger parts of the Earth's crust, thick tholeiitic volcanic sequences with associated large basic layered intrusions are found only in areas of tensional tectonics<sup>35</sup>, for example, Skaergaard, Duluth, and Scottish Tertiary complexes. The similarity of the younger examples to these older rocks suggests the Matagami-Chibougamau volcanic belt also formed in a tensional environment.

## Implications

The ores at Matagami are typical Archean Noranda-type deposits being (1) polymetallic (Fe-Zn-Cu-Ag-Au) massive sulphides of volcanogenic exhalative origin, (2) stratiform in configuration, and (3) associated with rhyolites near the tops of thick sequences of essentially basic submarine volcanic rocks<sup>36</sup>. In form and origin they are identical to younger volcanogenic types, but in metal content or in type of associated volcanic rock they may differ<sup>37,38</sup>. The younger deposits are widely regarded as falling into two major classes: (1) Fe-Cu-rich ores in basalts of tholeiitic affinity in tensional tectonic settings (Cyprus-type: spreading ridge; Besshi-type: back arc), and (2) Fe-Zn-Cu-Pb ores in rhyolite and dacite of calc-alkaline affinity developed along consuming plate margins (Kuroko-type)<sup>36,39,40</sup>. Although Noranda-type deposits contain only minor or trace amounts of Pb, they have been compared most frequently with the Kuroko-type because of their association with rhyolites and the apparent calc-alkaline nature of the volcanic sequences. Consequently, geological thought and exploration strategy in shield areas have been unduly preoccupied with the 'calc-alkaline' association of these ores, and by analogy, with compressional zones in the Earth's crust.

However, genesis of these massive sulphide ores is intimately related to sub-sea floor geothermal activity<sup>11,41-43</sup> during which Fe, Mg, Cu, Zn and many other elements are leached from large volumes of the underlying volcanic rocks and deposited at the sediment-seawater interface. It is these hydrothermally altered volcanic rocks, whose <sup>87</sup>Sr/<sup>86</sup>Sr, <sup>18</sup>O/<sup>16</sup>O and <sup>34</sup>S/<sup>32</sup>S ratios<sup>42,43</sup> and major and trace element geochemistry<sup>11,12</sup> have been so strongly disturbed, that are often described as 'calc-alkaline'. The apparent 'calc-alkaline' affinity of hydrothermally altered tholeiitic volcanic rocks in many mining districts may thus be the inevitable consequence of sub-seafloor geothermal activity, as will be outlined elsewhere.

Thus, Noranda-type massive sulphide deposits do occur in association with tholeiitic volcanic rocks, both here in Matagami, in the Flin Flon-Snow Lake mining district<sup>44</sup>, and apparently in several other districts<sup>45</sup>. This and other evidence also indicates they formed in tensional environments in the Earth's crust. Therefore, we conclude that sequences of submarine volcanic rocks containing rhyolites, whether of tholeiitic or calc-alkaline affinity, are prime exploration targets for Noranda-type massive sulphide ores.

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# Genetic variance of laboratory outbred Swiss mice

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*The extent of allelic variation has been estimated at 46 structural gene loci within three major colonies of Swiss mice and between inbred derivative strains. The colonies have retained nearly the same amount and type of variation found in natural murine or human populations despite laboratory propagation for more than 50 years (175 generations). The population genetic structures of the Swiss mouse colonies were comparable to an island population in which random fixation, and not inbreeding or population bottlenecks, is apparently responsible for slight losses in genetic variance.*

RODENTS provide a general standard in toxicological and carcinogenesis testing<sup>1,2</sup> and laboratory mice (*Mus musculus*) are especially useful because they are inexpensive, available in large number and have short lifespans. A major advantage is the availability of numerous inbred strains homozygous for over 98% of their genetic loci. In addition, considerable genetic information about the differences between strains has accumulated from research on mouse genetics, oncology and toxicology<sup>3–5</sup>. However, the divergence between inbred strains is now a source of criticism of their use for estimating human risk<sup>1,2</sup>. The problem stems from the expression of certain alleles which influence response to chemicals in various inbred strains, many of which were specifically selected to respond to particular chemicals or to have a high spontaneous incidence of tumours<sup>3–6</sup>. Thus, some toxicology testing protocols now involve laboratory outbred mice, on the rationale that such strains more closely resemble genetically heterogeneous human populations.

A key assumption in the use of outbred stocks is their genetic similarity to outbred mouse or human populations. Festing<sup>7</sup> has argued that the variation within an outbred stock is considerably smaller than that between inbred strains and has proposed the use of a panel of different inbred strains in a factorial design<sup>7,8</sup>. The true extent of gene variability in laboratory outbred stocks is unknown<sup>8–10</sup>, and no data have been reported on stocks of the Swiss mouse, the most commonly used outbred mouse line.

We have applied the techniques and concepts of population genetics to the biochemical genetic characterisation of outbred Swiss mice from three major production colonies known to supply a large proportion of such mice for carcinogenesis and toxicological testing. The extent of gene–enzyme electrophoretic variation at 46 structural genes is presented in addition

to the typing of several inbred Swiss strains. The results are analysed with respect to the following questions: (1) Do different isolated Swiss mouse colonies differ from each other in their population genetic structure? (2) Do outbred Swiss mice contain the same amount of variation observed in wild mouse populations, or has it been diminished by inbreeding, possibly through bottlenecks or by protection from the selective pressures of the wild state? (3) Is variation in Swiss mice qualitatively different (that is, with different variable loci, alleles or allele frequencies) from that in wild populations? In addition, the results of the analysis are related to considerations of population genetic theory of isolated populations.

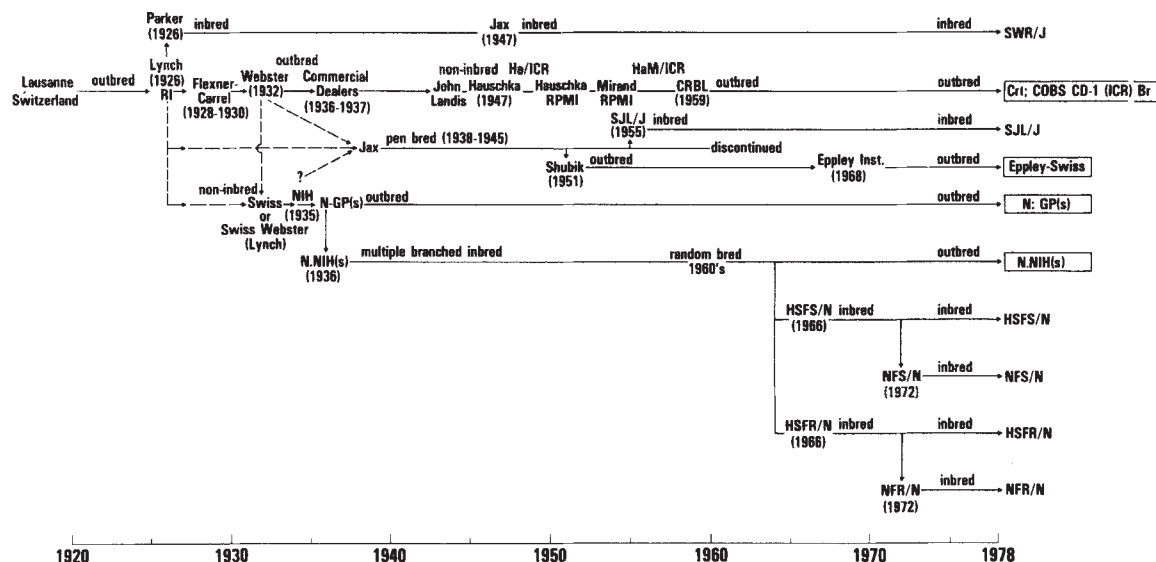
## History of the three Swiss mouse colonies

The original colony of Swiss mice in the US started from nine mice brought from Lausanne, Switzerland, in 1926 by Clara Lynch. Lynch<sup>11</sup> documented the genealogical history of the outbred strain starting from the Pasteur Institute in Paris until it reached the Rockefeller Institute where Webster maintained and dispersed Swiss and Swiss–Webster mice to researchers and commercial dealers. The three major colonies diverged from the Rockefeller colony in the late 1930s. The derivation of the outbred and inbred Swiss mouse strains is reconstructed in Fig. 1.

## An estimate of gene–enzyme variation in Swiss mice

Between 40 and 50 6-week-old female non-sibling Swiss mice were obtained from each of the three colonies in addition to several mice from the Swiss inbred lines, from BALB/c and from C57BL/6. Extracts prepared from kidney, liver, red blood cells and plasma from each animal were subjected to gel electrophoresis and developed histochemically for 26 enzymes and haemoglobin. The 27 systems were selected from isozyme systems used in our laboratory for studies in somatic cell genetics<sup>12–14</sup>. The extent and character of allozyme (allelic isozyme) variation at each of 46 loci (Table 1) were determined by comparing the allozyme phenotype with the enzyme mobilities obtained from BALB/c or C57BL/6 mice standards run in the same gel<sup>15–17</sup>. The utility of these systems in somatic cell genetics does not depend on allozyme polymorphism, and thus minimises the bias of published systems which are mostly polymorphic<sup>18</sup>. Of the 46 loci, 38 have been mapped to specific chromosomal sites in the mouse (Table 1), indicating that they are genetically autonomous of each other.

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**Fig. 1** Genealogical history of Swiss outbred mice and inbred derivatives. **CD1 colony:** In 1947, Hauschka established the Ha/ICR outbred stock from 100 female and 30 male 'Swiss-Webster' mice obtained from John Landis in Hagerstown, Maryland<sup>47</sup>. The origin of Landis' stock is obscure. When Hauschka moved from the Institute of Cancer Research, Philadelphia, to Roswell Park Memorial Institute, he passed some of the mice to E. A. Mirand. In 1957, Mirand sent 50 female mice to Charles River Breeding Laboratories where they founded the Cr1: COBS CD-1 (ICR) BR colony (G. Pucak, personal communication). The CD-1 stock is now bred on a 1 × 1 system adopting the 'Poiley method', a systematic circular method used to maximise outbreeding. A protected nucleus colony of 300–400 breeders is kept in sterile plastic film isolators and several production colonies produce 10,000–12,000 CD-1 pups each per week. To maintain homogeneity, pups from different production colonies are added to the nucleus periodically. **Eppléy-Swiss colony:** The Jackson Laboratory, Bar Harbor, received Swiss and Swiss-Webster outbred mice from three sources (C. Lynch, L. Webster and one unknown) between 1938 and 1945 (ref. 3) (J. Staats, J. Dorey, personal communications). These outbred lines were pen bred until 1955. In 1951, P. Shubik, Chicago Medical School, obtained Swiss mice from the pen-bred colony (B. Toth, personal communication) and started a closed, outbred colony. Shubik moved the colony to the Eppléy Institute, Omaha, Nebraska, in 1968, where it is now bred on a 1 × 1 scheme, using an extensive labelling system to insure random breeding in an isolated breeder colony of 150–180 pairs. The Eppléy-Swiss colony produces 18,000–26,000 mice per yr. **N.NIH(s) colony:** In 1935 the NIH received an undetermined number of outbred Swiss mice from the Rockefeller Institute that were the founders of the general purpose colony N: GP(s). In 1936, the N.NIH(s) was derived from the N: GP(s) colony. The N.NIH(s) colony was bred from 1936 to the 1960s by a multiple-branched inbreeding system described as a "brother-sister mating without following any systematic plan" (ref. 40). In the early 1960s a strict random mating scheme was resumed for the N.NIH(s) stock (C. T. Hansen, personal communication). The N.NIH(s) stock was rederived in 1972 by hysterectomy and foster nursing of mice to establish a specific pathogen-free (SPF) colony<sup>48</sup>. This nucleus colony consists of 120 pairs, is bred by a circular pair mating system and is selected for high productivity (C. T. Hansen, personal communication). The N.NIH(s) colony produces 140,000 mice per yr and the N: GP(s) colony 110,000 per yr. **Inbred strains:** In 1926, an inbred derivative of Lynch's Swiss strain, SWR, was established by Raymond Parker at the University of Toronto. In 1947, the SWR line was supplied to the Jackson Laboratory which continued the inbred line as SWR/J. The SJL/J strain was derived from the pen-bred Swiss strain at the Jackson Laboratory around 1955 when the outbred line was discontinued. In 1966, two inbred lines were derived from the N.NIH(s) colony. These lines were selected for their reaction to histamine after treatment with *Bordetella pertussis* and were thereby named HSFR/N (histamine-sensitive-factor resistant) and HSFS/N (histamine-sensitive-factor sensitive). In 1972, two new inbred lines, NFS/N and NFR/N, were rederived from HSFS/N and HSFR/N, respectively. During derivations of the present colonies, there were various periods of bottlenecks of unconfirmed size as well as periodic artificial selection of each outbred stock for fecundity (refs 11, 40, 47 and G. Pucak, J. Staats, J. Dorey, C. T. Hansen, personal communications). The following abbreviations were used: Jax: the Jackson Laboratories, Bar Harbor, Maine; RPMI: Roswell Park Memorial Institute, Buffalo, New York; NIH: NIH, Bethesda, Maryland; CRBL: Charles River Breeding Labs, Wilmington, Massachusetts; RI: Rockefeller Institute, New York, New York.

The genetic constitution of the three outbred mouse populations and six inbred derivative strains is presented for each locus in Table 1. Of the 46 loci tested, 37 (80.4%) were monomorphic in each population and, interestingly, for the same allele in all three colonies. Conversely, 9 of the 46 loci (19.6%) were polymorphic for at least two alleles in one or more of the three outbred colonies. Alternative alleles were found to be homozygous in different inbred strains at each of the polymorphic loci except *Gpi-1*. In addition, two of the loci which were invariant within the outbred colonies (*Es-3* and *Es-6*) were fixed for different alleles in derivative inbred strains. The finding of differential fixation for each polymorphic allele in different inbred derivatives is taken as evidence that the observed variation has a genetic basis, and that the present polymorphisms probably existed in and are thus derived from Lynch's original colony.

The allelic frequencies of each of the nine polymorphic loci in the three outbred mouse colonies are presented in Table 2. Six of the nine loci are polymorphic in all three populations, and three loci, *Gpi-1*, *Id-1* and *Got-2*, have become monomorphic or nearly so in at least one of the three colonies. The allelic frequencies were used to estimate the expected frequency of heterozygotes ( $2pq$ ) for each population at each polymorphic locus<sup>18–20</sup> also presented in Table 2. Each locus was tested for

conformity to the Hardy-Weinberg equilibrium (HWE) by a  $\chi^2$  test with one degree of freedom. Two departures from expectation for a random breeding population were observed: *Hbb* ( $\chi^2 = 16.45$ ,  $P \leq 0.0005$ ) and *Id-1* ( $\chi^2 = 7.6$ ,  $0.005 \leq P \leq 0.01$ ), both in the Eppléy population. Similar distortions of *Hbb* genotypic frequencies have been reported in several wild mouse populations<sup>21–24</sup>. The conformity of 19 of 20 tested loci (excluding *Hbb*) to HWE effectively excludes any marked influence of assortive mating, heterosis, migration or selection on the operation of random mating in these populations. As HWE is a stable one (it depends on allele frequencies at the time of sampling and is independent of allele frequencies in previous generations), the sample frequencies give little indication of the randomness (or otherwise) of mating in the 50-yr history of Swiss strains.

## Comparison of Swiss colonies to wild mouse populations

The proportion of polymorphic loci measured in the Swiss colonies and a number of feral mouse populations is presented in Table 3. Also presented for each population is the average heterozygosity, which is a better estimate of genic variation in random breeding populations because it takes gene frequencies



into account. The Eppley colony is the most polymorphic of the Swiss mice, with 19.6% of its loci polymorphic, compared with 17.4% CD-1, and 13.4% N.NIH(s). The average, 16.8%, is not quite as high as the average calculated for the natural mouse populations listed in Table 3, including two California populations (Lake Casitas and Bouquet Canyon) which were tested for the same 46 loci<sup>24</sup>. The average heterozygosity of Swiss mice is 0.071, which is only slightly lower than the average heterozygosity calculated for the two California feral mouse populations tested for the same 46 loci (0.084) and previously published estimates for mice (Table 3). The values are also comparable to variability estimates of human populations reported by Harris and Hopkinson (0.067)<sup>25</sup> and Nei and Roychoudhury (0.10)<sup>26</sup>.

The similarity between the three Swiss colonies and feral mice tested in this laboratory was quantitated using Nei's genetic

distance<sup>27,28</sup> (Table 4). This statistic measures the degree of allelic substitution between populations based on electrophoretic mobility of several gene-enzyme systems. Within the limits of Nei's assumptions<sup>27</sup>, the genetic distance estimates increase proportionately with the amount of time the populations have been reproductively isolated. The average distance between the three Swiss colonies is slight (0.023), approximately the same as the distance between the Lake Casitas and Bouquet Canyon (0.019) which are local populations separated by 40 miles in southern California<sup>24</sup>. The genetic distance between the Swiss mice and the California mice (average 0.062) is three times the distance within the two groups, indicating an isolation of Swiss from American mice three times longer than isolation within either group. This observation is consistent with the European origin of Swiss mice some 70 years ago.

Table 1 Allozyme composition of Swiss outbred mice populations and derivative inbred mouse strains

| Gene symbol       | Outbred  |      |              | Inbred derivatives |       |     |     |      |      |
|-------------------|----------|------|--------------|--------------------|-------|-----|-----|------|------|
|                   | N.NIH(s) | CD-1 | Eppley-Swiss | SWR/J              | SJL/J | NFS | NFR | HSFS | HSFR |
| <i>Acp-1</i> (12) | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Ada</i>        | A        | A    | A            | A                  | A     | A   | A   | A    | A    |
| <i>Apri</i> (8)   | A        | A    | A            | A                  | A     | A   | A   | A    | A    |
| <i>Ak-1</i> (2)   | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Ak-2</i> (4)   | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Adh-2</i>      | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Es-1</i> (8)   | b        | b    | b            | b                  | b     | b   | b   | b    | b    |
| <i>Es-2</i> (8)   | b        | b    | b            | b                  | b     | b   | b   | b    | b    |
| <i>Es-3</i> (11)  | c        | c    | c            | c                  | c     | a   | c   | c    | c    |
| <i>Es-5</i> (8)   | b        | b    | b            | —                  | —     | b   | b   | b    | b    |
| <i>Es-6</i> (8)   | a        | a    | a            | b                  | b     | a   | a   | a    | a    |
| <i>Es-10</i> (14) | a        | a    | a            | a                  | a     | a   | a   | a    | a    |
| <i>Es-s</i>       | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>G6PD</i> (X)   | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Gpi-1</i> (7)  | b        | a, b | a, b         | b                  | a     | b   | b   | b    | b    |
| <i>Got-1</i> (19) | a        | a    | a            | a                  | —     | a   | a   | a    | a    |
| <i>Got-2</i> (8)  | a, b     | a, b | a, b         | a                  | b     | a   | a   | a    | a    |
| <i>Gpt-1</i> (15) | a, c     | a, c | a, c         | a                  | a     | a   | a   | a    | a    |
| <i>Gapdh</i>      | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Gr-1</i> (8)   | a, b     | a, b | a, b         | b                  | b     | b   | b   | a    | b    |
| <i>Glo-1</i> (17) | a, b     | a, b | a, b         | —                  | a     | a   | a   | a    | b    |
| <i>Hba</i> (11)   | A        | A    | A            | A                  | A     | A   | A   | A    | A    |
| <i>Hbb</i> (7)    | s, d     | s, d | s, d         | s                  | s     | d   | d   | d    | d    |
| <i>Hk-1</i> (10)  | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Hprt</i> (X)   | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Id-1</i> (1)   | a, b     | a    | a, b         | a                  | b     | a   | a   | a    | a    |
| <i>Id-2</i> (7)   | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Ldh-1</i> (7)  | A        | A    | A            | A                  | A     | A   | A   | A    | A    |
| <i>Ldh-2</i>      | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Mor-1</i> (5)  | a        | a    | a            | —                  | —     | a   | a   | a    | a    |
| <i>Mor-2</i>      | A        | A    | A            | A                  | A     | A   | A   | A    | A    |
| <i>Mod-2</i> (7)  | b        | b    | b            | b                  | b     | b   | b   | b    | b    |
| <i>Mod-1</i> (9)  | a, b     | a, b | a, b         | a                  | a     | a   | a   | a    | b    |
| <i>Mpi-1</i> (9)  | b        | b    | b            | b                  | b     | b   | b   | b    | b    |
| <i>Np-1</i> (14)  | a        | a    | a            | a                  | a     | a   | a   | a    | a    |
| <i>Pep-1</i> (18) | A        | A    | A            | A                  | A     | A   | A   | A    | A    |
| <i>Pep-2</i> (10) | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Pep-3</i> (1)  | b        | b    | b            | b                  | b     | b   | b   | b    | b    |
| <i>Pep-4</i> (7)  | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Pep-7</i> (5)  | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Pgd-1</i> (4)  | b        | b    | b            | b                  | b     | b   | b   | b    | b    |
| <i>Pgm-1</i> (5)  | a, b     | a, b | a, b         | b                  | b     | a   | b   | a    | a    |
| <i>Pgm-2</i> (4)  | a        | a    | a            | a                  | a     | a   | a   | a    | a    |
| <i>Pyp</i> (10)   | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Sod-1</i>      | A        | A    | A            | —                  | —     | A   | A   | A    | A    |
| <i>Sod-2</i>      | A        | A    | A            | —                  | —     | A   | A   | A    | A    |

The following enzymes were scored from mouse liver extracts: acid phosphatase (*Acp-1*), adenosine deaminase (*Ada*), glucose-6 phosphate dehydrogenase (*G6PD*); from red blood cell lysates: esterase-3 (*Es-3*), esterase-10 (*Es-10*), glucose phosphate isomerase (*Gpi-1*), haemoglobin  $\alpha$ ,  $\beta$  chain (*Hba*, *Hbb*), phosphoglucosyltransferase-1 and -2 (*Pgm-1* and *Pgm-2*); from serum: esterase-5 (*Es-5*), esterase-s (*Es-s*); from kidney: adenine phosphoribosyl transferase (*Apri*), adenylate kinase-1 (*Ak-1*), adenylate kinase-2 (*Ak-2*), alcohol dehydrogenase (*Adh*), esterase-1 (*Es-1*), esterase-2 (*Es-2*), esterase-6 (*Es-6*), glutamate oxaloacetate transaminase(soluble) (*Got-1*), glutamate oxaloacetate transaminase(mitochondrial) (*Got-2*), glutamate pyruvate transaminase(soluble) (*Gpt-1*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), glutathione reductase (*Gr-1*), glyoxylase (*Glo-1*), hexokinase (*Hk-1*), hypoxanthine phosphoribosyl transferase (*Hprt*), isocitrate dehydrogenase(soluble) (*Id-1*), isocitrate dehydrogenase(mitochondrial) (*Id-2*), lactate dehydrogenase-A (*Ldh-1*), lactate dehydrogenase-B (*Ldh-2*), malate dehydrogenase(mitochondrial) (*Mor-1*), malate dehydrogenase(soluble) (*Mor-2*), malic enzyme(mitochondrial) (*Mod-2*), malic enzyme(soluble) (*Mod-1*), mannose phosphate isomerase (*Mpi-1*), purine nucleoside phosphorylase (*Np-1*), peptidase-1 (*Pep-1*), peptidase-2 (*Pep-2*), peptidase-3 (*Pep-3*), peptidase-4 (*Pep-4*), peptidase-7 (*Pep-7*), 6-phosphogluconate dehydrogenase (*Pgd-1*), pyrophosphatase (*Pyp*), superoxide dismutase(soluble) (*Sod-1*), superoxide dismutase(mitochondrial) (*Sod-2*). The vertical starch gel electrophoresis apparatus (Buchler) was used in combination with 12% Electrostar (Electrostarch) for each system except adenosine and hypoxanthine-guanine-phosphoribosyl transferase. These systems were assayed following electrophoresis in 7% acrylamide in an EC-Apparatus (E.C. Apparatus). Phosphoribosyl transferases were detected by incubation of the gels with <sup>14</sup>C-labelled substrates. The phosphorylated products were precipitated with LaCl<sub>3</sub>, and the gels were dried and exposed to autoradiography<sup>41</sup>. Haemoglobin was separated in gels containing 0.8% cystamine dihydrochloride<sup>42</sup>. The remaining enzymes examined were separated and visualised by modifications of standard isozyme procedures<sup>43-45</sup>. A = no name assigned to this allele. Numbers in parentheses after gene symbol are chromosome assignments.

Table 2 Polymorphic loci of Swiss mice

| Enzyme<br>(chromosome) | N.NIH(s)             |      |                 | CD-1                 |      |                 | Eppley-Swiss         |      |                 |
|------------------------|----------------------|------|-----------------|----------------------|------|-----------------|----------------------|------|-----------------|
|                        | Allele<br>frequency  | 2pq  | HWE<br>$\chi^2$ | Allele<br>frequency  | 2pq  | HWE<br>$\chi^2$ | Allele<br>frequency  | 2pq  | HWE<br>$\chi^2$ |
| Gpi-1 (7)              | b = 1.0              | —    | —               | a = 0.43<br>b = 0.57 | 0.49 | 2.1             | b = 0.40<br>a = 0.60 | 0.48 | 1.11            |
| Gr-1 (8)               | a = 0.35<br>b = 0.65 | 0.46 | 0.85            | a = 0.56<br>b = 0.44 | 0.49 | 1.8             | a = 0.47<br>b = 0.53 | 0.50 | 0.31            |
| Got-2 (8)              | b = 0.97<br>a = 0.03 | 0.06 | —               | b = 0.60<br>a = 0.40 | 0.48 | 0.39            | b = 0.27<br>a = 0.73 | 0.39 | 2.76            |
| Gpt-1 (15)             | a = 0.67<br>b = 0.33 | 0.44 | —               | a = 0.80<br>b = 0.20 | 0.32 | —               | a = 0.57<br>b = 0.43 | 0.49 | —               |
| Glo-1 (17)             | a = 0.64<br>b = 0.36 | 0.46 | 0.38            | a = 0.86<br>b = 0.14 | 0.24 | 2.36            | a = 0.62<br>b = 0.38 | 0.47 | 0.24            |
| Hbb (7)                | s = 0.56<br>d = 0.44 | 0.49 | 0.64            | s = 0.82<br>d = 0.18 | 0.30 | 1.86            | s = 0.40<br>d = 0.60 | 0.48 | 16.45           |
| Id-1 (1)               | a = 0.97<br>b = 0.03 | 0.06 | —               | a = 1.0              | —    | —               | a = 0.70<br>b = 0.30 | 0.42 | 7.6             |
| Mod-1 (9)              | b = 0.12<br>a = 0.88 | 0.21 | 0.70            | b = 0.83<br>a = 0.17 | 0.28 | 0.74            | b = 0.27<br>a = 0.73 | 0.39 | 2.17            |
| Pgm-1 (5)              | a = 0.37<br>b = 0.63 | 0.47 | 3.85            | a = 0.57<br>b = 0.43 | 0.49 | 1.45            | a = 0.64<br>b = 0.36 | 0.46 | 2.69            |

2pq is the predicted frequency of heterozygotes based on allele frequencies. HWE  $\chi^2$  is the value obtained in testing conformity of observed phenotype frequencies to those expected in conditions of Hardy-Weinberg equilibrium.

The Swiss outbred mouse polymorphisms are not qualitatively different from those reported for wild mouse populations<sup>29-32</sup>. Eight of the nine polymorphic loci (excluding *Glo-1*)<sup>33</sup> seen in Swiss outbred mice vary between inbred strains<sup>15-17</sup> and in wild mouse surveys. Of the 37 gene-enzyme systems which are monomorphic in Swiss outbred mice, 9 (*Es-1*, *Es-2*, *Es-3*, *Es-5*, *Mor-1*, *Pep-2*, *Pgd-1*, *Pgm-2* and *Sod-1*) were polymorphic in at least one natural population previously studied, but only 3 (*Es-2*, *Es-3*, *Pep-2*) were polymorphic in the majority of the natural populations sampled<sup>23,29-32</sup>. The other gene enzyme systems were either monomorphic or never tested in natural populations.

## Have the Swiss colonies been supplemented by non-Swiss mice?

It is, unfortunately, impossible to determine whether or not any of the Swiss stocks has been contaminated with non-Swiss mice during their history. Our data do, however, strongly suggest that migration from non-Swiss mice significant enough to change the genetic constitution of the stock has not occurred in any of the three colonies. First, the genetic distance (Table 4) between the colonies is small and approaches the limits of population identity provided by this statistic<sup>27,28</sup>. The inter-colony distances are lower than 140, distances calculated between 18 different feral mice populations previously studied (S. J. O'Brien, in preparation). Second, two of the loci which were polymorphic in Swiss mice, *Gpi-1* and *Pgm-1*, are monomorphic in American populations<sup>24,29</sup>, where the colonies developed, and polymorphic in European mice, the purported founders of Swiss mice. Third, *Glo-1* is monomorphic in wild mice<sup>24</sup>, and between all inbred strains (excluding HSFR and MA/MyJ, Swiss derivatives<sup>33</sup>), but is polymorphic at appreciable frequencies in each of the Swiss colonies. Fourth, the Swiss inbred strains (Table 1) are fixed for each of the different alleles at each locus polymorphic in the outbred colonies. These genetic distributions are characteristic of each colony and thereby emphasise their consanguinity with each other and their distinctiveness from other mouse populations which might have been a source of migrating genes.

## Swiss mice as an island population

The Swiss mouse has been isolated from its natural environment for over 50 yr (approximately 175 generations) and the colonies studied have been separated for over 30 yr (~122 generations).

If, as claimed by their keepers, they have not been supplemented by non-Swiss mice during this period, they can be considered as characteristic island populations<sup>34</sup>, isolated from migration and the selective rigours of the wild state. The retention of considerable genic variability comparable to that of mainland populations has been observed in feral mice in Hawaii<sup>21</sup> and Skokholm Island off the British mainland<sup>31,32</sup>, and in six island populations of *Drosophila willistoni* in the West Indies<sup>35</sup>. In addition, at least one laboratory population of *Drosophila*

Table 3 Proportion of loci estimated to be polymorphic and the proportion of the genome estimated to be heterozygous in mouse populations

| Populations                      | No. of<br>loci | No. of<br>populations | Proportion of<br>polymorphic<br>loci (%) | Average<br>hetero-<br>zygosity | Refs       |
|----------------------------------|----------------|-----------------------|------------------------------------------|--------------------------------|------------|
| Swiss laboratory mice            |                |                       |                                          |                                |            |
| N.NIH(s)                         | 46             | 1                     | 13.4                                     | 0.056                          | This study |
| CD-1                             | 46             | 1                     | 17.4                                     | 0.067                          | This study |
| Eppley-Swiss                     | 46             | 1                     | 19.6                                     | 0.089                          | This study |
| Average                          | 46             | 3                     | 16.8                                     | 0.071                          | This study |
| Wild feral mice                  |                |                       |                                          |                                |            |
| Lake Casitas, Calif.             |                |                       |                                          |                                |            |
| <i>Mus musculus domesticus</i>   | 46             | 1                     | 24                                       | 0.099                          | 24         |
| Bouquet Canyon, Calif.           |                |                       |                                          |                                |            |
| <i>Mus musculus domesticus</i>   | 46             | 1                     | 17                                       | 0.077                          | 24         |
| Hallowell Farm, Calif.           |                |                       |                                          |                                |            |
| <i>Mus musculus brevisrostri</i> | 40             | 10                    | 30                                       | 0.11                           | 29         |
| Denmark                          |                |                       |                                          |                                |            |
| <i>Mus musculus musculus</i>     | 41             | 4                     | 29                                       | 0.087                          | 30         |
| Denmark                          |                |                       |                                          |                                |            |
| <i>Mus musculus domesticus</i>   | 41             | 2                     | 22                                       | 0.070                          | 30         |
| British mainland                 | 22             | 6                     | 29                                       | 0.065                          | 31         |
| British Islands                  | 22             | 20                    | 17                                       | 0.045                          | 31         |
| Balenya, Spain                   |                |                       |                                          |                                |            |
| <i>Mus hispanicus (spretus)</i>  | 56             | 1                     | 20                                       | 0.034                          | 46         |

*melanogaster* retained levels of polymorphism nearly identical to those of wild populations after laboratory propagation for over 20 yr (520 generations)<sup>36</sup>.

The slight loss in genetic variance we observe here compared with wild mice or previous variability in Swiss populations indicated by variant inbred mice at two additional (to presently polymorphic) loci is probably due to genetic drift. The following calculation tends to support this notion. To estimate the rate of lost variation due to drift in isolated populations, Fisher<sup>37</sup> and Wallace<sup>38</sup> derived the equation

$$H_t = H_0 e^{-t/2N}$$

where  $H_t$  is the proportion of heterozygotes at generation  $t$ ,  $H_0$  is the proportion of heterozygotes at generation 0, and  $N$  is the



effective breeding size of the population. We can estimate the minimum average breeding size ( $N$ ) of the three colonies which would result in the observed average heterozygosity by estimating  $H_0$  from data for either inbred mice or wild mice as a norm over 175 (+) generations in the laboratory. If  $H_0 = 0.12$  (assuming 11/46 heterozygous loci originally, at a maximum heterozygosity of 0.5 at each locus), then  $N = 118$  for N.NIH(s), 154 for CD-1 and 293 for Eppley. If we use  $H_0 = 0.10$  (from the highest mouse population, Lake Casitas, in Table 3), the  $N$  values become 138 for NIH(s), 201 for CD-1 and 665 for Eppley. Thus, one would expect the average heterozygosity seen in the Swiss mice from genetic drift alone if their breeding sizes approximated the calculated values. With smaller breeding sizes the genetic variance would be less than we observe. The actual breeding sizes of the colonies are clearly well within these predicted ranges. In fact, one generation bottlenecks (for example, Lynch's original transport or caesarian rederivations of Swiss mice) of as few as 10 mice would have resulted in the loss of less than 10% of the included variation<sup>39</sup>. From these considerations, the possibility of inbreeding (conscious or not) to the extent of significantly diminishing the genetic variance of Swiss mice is unlikely.

## Conclusions

The results of this survey of three isolated colonies of Swiss mice indicate that a considerable amount of genetic variation persists in the stock. The average heterozygosity of Swiss mice (0.07) is very similar to estimates for feral mouse<sup>24,29-32</sup> and human<sup>25,26</sup> populations. Sampling of six inbred mouse strains derived at different periods in the history of Swiss mice provided the equivalent of fixed gametic samples from the generation of origin of the inbred line. The inbred samplings reinforce the age and persistence of the presently observed polymorphisms and indicate the existence of variability at two loci now monomorphic in Swiss mice. The population structure of the three colonies today is consistent with prior fixation at these loci due to genetic drift, possibly during population bottlenecks or short periods of inbreeding<sup>11,40</sup>. The populations today appear to be functionally random bred insofar as their genotype frequencies distribute as a binomial Hardy-Weinberg equilibrium.

With respect to the three questions posed at the outset, the data are quite specific: (1) The three Swiss colonies are quite similar to each other in the extent and character of their included genic variation. The genetic distance between colonies is low, as would be predicted of populations separated for a few hundred generations. (2) The Swiss mice contain slightly less variation than do wild mouse populations, but the similarities to feral

populations are more apparent than the differences. (3) The alleles observed, both monomorphic and polymorphic, are for the most part the same ones found in wild populations.

With reference to the question of efficacy of using outbred mice in carcinogenesis and toxicology protocols, the data presented here are precise, although specifically limited. The Swiss mouse colonies do seem to provide a realistic model of isolated outbred mouse populations in terms of persistence of genetic variation. If one chooses to test outbred animals, these colonies will suffice. Thus, the argument that random-bred laboratory strains are probably inbred seems to be incorrect.

**Table 4** Genetic distance ( $D$ ) and identity ( $I$ ) estimates of Swiss and wild mouse populations

|                 | $I$          |                |          |                 |        |
|-----------------|--------------|----------------|----------|-----------------|--------|
|                 | Lake Casitas | Bouquet Canyon | N.NIH(s) | CD <sub>1</sub> | Eppley |
| Lake Casitas    |              | 0.981          | 0.928    | 0.939           | 0.969  |
| Bouquet Canyon  | 0.019        |                | 0.922    | 0.931           | 0.949  |
| N.NIH(s)        | 0.075        | 0.081          |          | 0.976           | 0.975  |
| CD <sub>1</sub> | 0.063        | 0.071          | 0.025    |                 | 0.980  |
| Eppley          | 0.032        | 0.052          | 0.025    | 0.020           |        |

$I$  equals the probability of allelic identity of any randomly selected genes at any locus in each of two test populations.  $D$  equals the average number of gene differences per locus between individuals from the two test populations. Algebraically

$$I = J_{xy} / \sqrt{J_x J_y}$$

$$D = -\ln I$$

where  $J_{xy}$  is the arithmetic mean of  $j_{xy} = \sum_i x_i y_i$  over all loci,  $J_x$  is the arithmetic mean of  $j_x = \sum_i x_i^2$  over all loci, and  $x_i$  (or  $y_i$ ) is the frequency of the  $i$ th allele in the first (or second) population<sup>28</sup>.

Nevertheless, the decision to use outbred as opposed to inbred animals for testing protocols must ultimately be made on the basis of the information desired from the individual test, considerations which are beyond the scope of this report.

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# Transglutaminase is essential in receptor-mediated endocytosis of $\alpha_2$ -macroglobulin and polypeptide hormones

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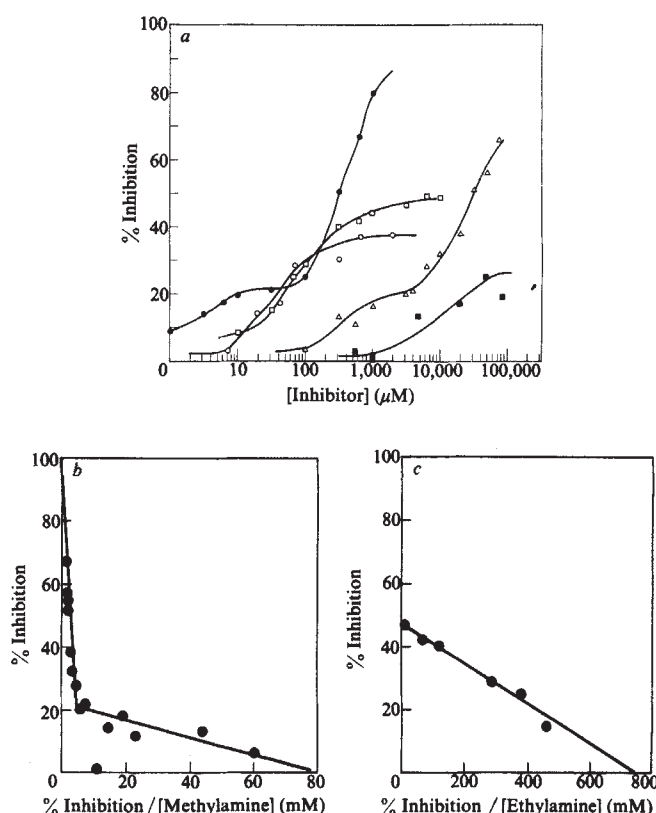
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*The receptor-mediated endocytosis of  $\alpha_2$ -macroglobulin can be inhibited by a diverse group of chemical compounds all of which share the property of being inhibitors of one form of cellular transglutaminase. The present results strongly suggest that protein cross-linking may be essential for receptor-mediated endocytosis of some protein and polypeptide hormones.*

RECEPTOR-mediated endocytosis is the process by which cells bind and internalise specific extracellular ligands. The process is responsible for the uptake of a variety of proteins and peptides, including the serum proteins low density lipoprotein (LDL)<sup>1</sup> and  $\alpha_2$ -macroglobulin ( $\alpha_2$ M)<sup>2-5</sup>, several lysosomal hydrolases<sup>6,7</sup> and the polypeptide hormones insulin and epidermal growth factor (EGF)<sup>8-12</sup>. For several of the ligands, ultrastructural studies have shown that internalisation occurs over clathrin-coated regions of the membrane<sup>1,4,11,12</sup>. The internalisation of  $\alpha_2$ M follows the same pathway as the internalisation of insulin and EGF<sup>2</sup>; unoccupied receptors are diffusely distributed on the cell surface of cultured fibroblasts and cluster over clathrin-coated pits after the ligand binds<sup>2,8,10</sup>. LDL receptors, on the other hand, seem to be concentrated over coated regions even in the absence of ligand binding<sup>1</sup>. These coated regions pinch off to form endocytotic vesicles which transfer the internalised ligands to other organelles.

Although the steps involved in receptor-mediated endocytosis have been described, the molecular mechanisms involved in the process are unknown. To understand the mechanism of ligand internalisation we have searched for inhibitors of receptor-mediated endocytosis. Recently, we have observed that the first step in the process, the aggregation of ligand-receptor complexes over coated pits, required extracellular  $\text{Ca}^{2+}$  and was inhibited by primary alkylamines<sup>13</sup>. This observation suggested several potential mechanisms of inhibition, including the possibility that a transglutaminase might be important in the process. Transglutaminase is an intracellular enzyme found in many types of cells. Some transglutaminases covalently cross-link proteins by forming  $\epsilon$ -( $\gamma$ -glutamyl)-lysine cross-bridges (for review see ref. 14). These enzymes require  $\text{Ca}^{2+}$  for activity and can be inhibited by a variety of primary amines. Several forms of transglutaminase are known. Guinea pig liver transglutaminase and plasma transglutaminase have been purified, and a wide variety of specific inhibitors of these enzymes have been identified<sup>14-16</sup>.

To test the hypothesis that a transglutaminase is involved in receptor-mediated endocytosis, we tested a wide variety of inhibitors of transglutaminases to see if they blocked ligand clustering and internalisation. We have found an excellent correlation between the ability of compounds to inhibit a



**Fig. 1** Inhibition of transglutaminase in CHO extracts. *a*, log dose-response curves for inhibition of CHO transglutaminase by dansylcadaverine (●), ethylamine (□), bacitracin (○), methylamine (Δ) and tolbutamide (■). *b*, Plot of fractional inhibition against fractional inhibition/inhibitor concentration (Eadie-Hofstee) for methylamine on CHO transglutaminase. *c*, As (*b*) but for ethylamine. CHO cells were grown in suspension culture in Dulbecco's modified essential media (DMEM) containing 10% heated calf serum to a density of  $1 \times 10^6$  cells per ml. Cells were pelleted, washed twice in phosphate-buffered normal saline (PBS), resuspended to 1–4 mg cell protein per ml in PBS and lysed by sonication. Extracts were frozen at  $-70^\circ\text{C}$  and then thawed and assayed for transglutaminase activity. Assays measured the  $\text{Ca}^{2+}$ -dependent incorporation of  $^3\text{H}$ -putrescine into casein using a filter paper assay<sup>18</sup>. The assay mixture (200  $\mu\text{l}$ ) contained 20 mM Tris-HCl, pH 7.4, 2 mg ml<sup>-1</sup> casein (Mann), 5 mM mercaptoethanol, 5 mM  $^3\text{H}$ -putrescine (40 c.p.m. pmol<sup>-1</sup> NEN) and various concentrations of inhibitor. All inhibitors were adjusted to pH 7.4 before addition to the assay. The reaction was initiated by addition of  $^3\text{H}$ -putrescine and incubation at  $37^\circ\text{C}$  and duplicate aliquots (25  $\mu\text{l}$ ) were taken at 3 and 6 min, spotted on filter papers (Whatman 3MM) and precipitated by addition to cold 10% trichloroacetic acid. After washing, dehydrating and drying<sup>18</sup>, filters were counted in a toluene-based scintillation cocktail (Econofluor, NEN) at an efficiency of 13%. The background activity measured in the absence of  $\text{Ca}^{2+}$  (5 mM EGTA) was 50–150 c.p.m. and was subtracted from all values. Enzyme activity varied over 4–10 nmol putrescine incorporated per min per mg cell protein, depending on the preparation used. Data shown represent means of duplicate determinations pooled from at least two separate experiments.

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fibroblast transglutaminase and their ability to inhibit the aggregation and internalisation of  $\alpha_2$ M. It therefore seems possible that protein cross-linking is essential for the endocytosis of hormones and  $\alpha_2$ -macroglobulin.

### Inhibition of fibroblast transglutaminase

Transglutaminase activity in fibroblasts was assayed by measuring  $\text{Ca}^{2+}$ -dependent covalent incorporation of  $^3\text{H}$ -putrescine into casein<sup>17,18</sup>. Transglutaminase activity (4–10 nmol per min per mg cell protein) was 10–20 times higher in extracts of CHO cells than in normal rat kidney (NRK) fibroblasts or BALB 3T3 fibroblasts. Therefore, we used CHO extracts to characterise inhibitors of fibroblast transglutaminase.

We first examined methylamine as an inhibitor of CHO transglutaminase. As shown in Fig. 1a, low concentrations of methylamine produced partial inhibition of CHO transglutaminase activity but much higher concentrations were required to produce complete inhibition. Kinetic analysis of the inhibition curve (Fig. 1b) showed that the inhibition consisted of two components. A high-affinity component ( $K_i$  0.2 mM) accounted for 25% of the total activity, and a low-affinity component ( $K_i$  20 mM) accounted for the remainder. The fraction of high-affinity component varied from 25 to 50% total activity depending on the preparation of cells used. Similar results were obtained with other fibroblast cell lines, NRK and BALB 3T3, but because of the low specific activity and a lower fraction of the high-affinity transglutaminase pool, quantitative measurements were difficult.

Ethylamine was more potent than methylamine (Fig. 1a), but even high concentrations produced only partial inhibition of transglutaminase activity. Kinetic analysis of the ethylamine inhibition curve (Fig. 1c) showed a straight line, indicating a single component of inhibition, with  $K_i$  35  $\mu\text{M}$ , that accounted for 45% of the total enzyme activity. *n*-Propylamine, isopropylamine and *n*-hexylamine were all equivalent to ethylamine as inhibitors of CHO transglutaminase (data not shown).

Dansylcadaverine, a very potent inhibitor of liver and serum transglutaminases<sup>16,19</sup>, is also a potent inhibitor of CHO transglutaminase (Fig. 1a). Concentrations of 1–100  $\mu\text{M}$  produced partial inhibition of the enzyme activity whereas higher concentrations produced complete inhibition. As with methylamine, kinetic analysis demonstrated two components of inhibition, a high-affinity component ( $K_i$  2.5  $\mu\text{M}$ , 28% total activity) and a low-affinity component ( $K_i$  200  $\mu\text{M}$ , 72% of total activity).

We have also identified two novel inhibitors of CHO transglutaminase activity, the peptide antibiotic, bacitracin, which blocks hormone degradation<sup>20</sup> and the sulphonylurea, tolbutamide. As shown in Fig. 1a, both produced partial inhibition of CHO transglutaminase; bacitracin had a  $K_i$  comparable to that of ethylamine, 35  $\mu\text{M}$ . Tolbutamide was much less active, the  $K_i$  being approximately 2 mM.

### Dansylcadaverine inhibition of receptor-mediated endocytosis in CHO cells

$\alpha_2$ -Macroglobulin binds to saturable cell-surface receptors<sup>5</sup>. As shown in Table 1, rhodamine-labelled  $\alpha_2$ M ( $\text{R-}\alpha_2\text{M}$ ) retains the ability to bind to these receptors. The bound  $\alpha_2$ M becomes concentrated over clathrin-coated pits<sup>4</sup> and is internalised in endocytotic vesicles<sup>21</sup>.

As the biochemical studies on transglutaminase were carried out in CHO cell extracts, we also characterised the clustering and internalisation of  $\alpha_2$ M by these cells. The experimental protocol was similar to that reported by Maxfield *et al.*<sup>13</sup>. CHO cells in serum-free culture media were exposed to  $\text{R-}\alpha_2\text{M}$  for 30 min at 37 °C. Unbound  $\text{R-}\alpha_2\text{M}$  was then removed and the cells were washed and fixed with formaldehyde. The bound  $\text{R-}\alpha_2\text{M}$  was viewed in an epifluorescence microscope using a silicon intensifier television camera<sup>22</sup>. Data presented are photographs of a video monitor.

Figure 2 shows receptor-mediated endocytosis of  $\text{R-}\alpha_2\text{M}$  by CHO cells and the effect of dansylcadaverine on the process. Figure 2a shows the punctuate pattern of fluorescence which is

**Table 1** Fluorescence intensities of NRK cells incubated with rhodamine- $\alpha_2$ -macroglobulin

| Incubation conditions                                                                                   | Fluorescence intensity (arbitrary units) |
|---------------------------------------------------------------------------------------------------------|------------------------------------------|
| $\text{R-}\alpha_2\text{M}$ (10 $\mu\text{g ml}^{-1}$ )                                                 | 4 $\pm$ 2                                |
| $\text{R-}\alpha_2\text{M}$ (30 $\mu\text{g ml}^{-1}$ )                                                 | 18 $\pm$ 6                               |
| $\text{R-}\alpha_2\text{M}$ (100 $\mu\text{g ml}^{-1}$ )                                                | 57 $\pm$ 16                              |
| $\text{R-}\alpha_2\text{M}$ (300 $\mu\text{g ml}^{-1}$ )                                                | 95 $\pm$ 20                              |
| $\text{R-}\alpha_2\text{M}$ (100 $\mu\text{g ml}^{-1}$ ) + $\alpha_2\text{M}$ (2 mg $\text{ml}^{-1}$ )* | 9 $\pm$ 4                                |
| Dansylcadaverine (50 $\mu\text{M}$ ), 30 min; $\text{R-}\alpha_2\text{M}$ (100 $\mu\text{g ml}^{-1}$ )† | 43 $\pm$ 12                              |
| SFM, 30 min; $\text{R-}\alpha_2\text{M}$ (100 $\mu\text{g ml}^{-1}$ )†                                  | 48 $\pm$ 15                              |

NRK cells were plated on Falcon tissue culture dishes and grown to confluency. The cells were rinsed with serum-free medium (SFM) and incubated with the indicated concentrations of  $\text{R-}\alpha_2\text{M}$  in SFM for 30 min at 37 °C. Cells were rinsed, fixed and observed as described in Fig. 2 legend. Clusters were observed at all concentrations of  $\text{R-}\alpha_2\text{M}$  tested. For intensity measurements, an EMI 9658 R photomultiplier tube was mounted on the microscope, and the incident illumination was passed through a monochromator (Instruments SA, model H10) with a spectral bandpass of 8 nm in addition to the narrow bandpass filter on the microscope. The diameter of the illumination beam was approximately 30  $\mu\text{m}$  in the focal plane. The values shown are the mean and standard deviation of the intensities at 20 randomly selected locations on the dish. Untreated cells had an intensity of 5 units and this was subtracted from all the values shown. Duplicate dishes gave the same average value  $\pm$  10%. Similar results were obtained with CHO cells. Trypsin treatment of the  $\text{R-}\alpha_2\text{M}$  to form trypsin- $\alpha_2\text{M}$  complexes<sup>5</sup> did not significantly alter the binding.

\*  $\alpha_2\text{M}$  was added to the cultures 5 min before  $\text{R-}\alpha_2\text{M}$ .

† Dansylcadaverine was added 30 min before  $\text{R-}\alpha_2\text{M}$ ; control dishes were incubated with SFM.

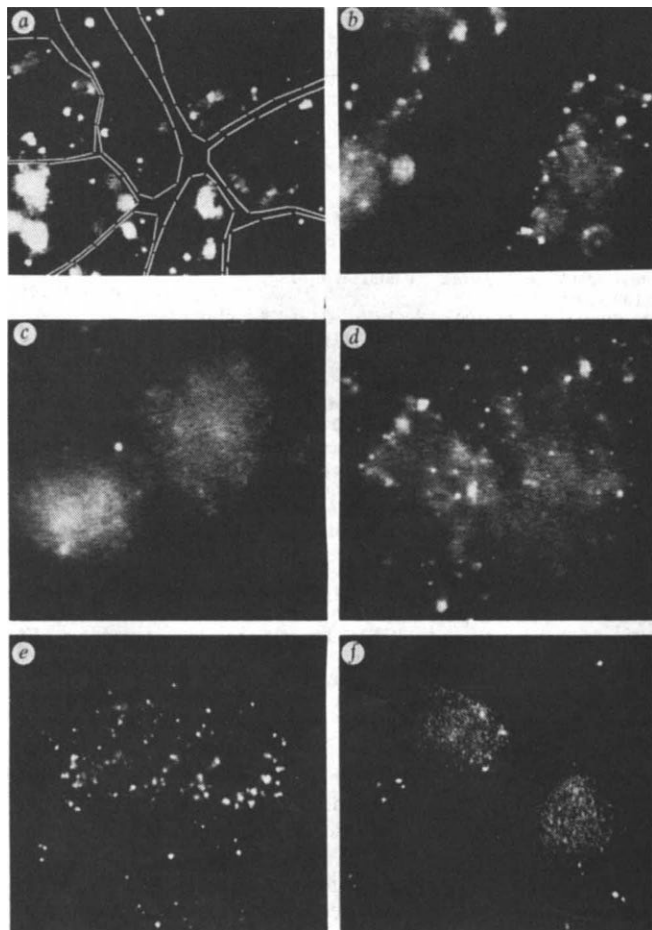
seen after a 30-min incubation with  $\text{R-}\alpha_2\text{M}$  at 37 °C. This pattern represents the result of the clustering and internalisation of the  $\text{R-}\alpha_2\text{M}$ . The dashed lines denote the margins of individual cells.

As can be seen in Fig. 2b, cells treated with 20  $\mu\text{M}$  dansylcadaverine show a marked reduction in the number and intensity of fluorescent spots. There is a compensating increase in diffusely distributed fluorescence. The effect is more marked with 100  $\mu\text{M}$  dansylcadaverine (Fig. 2c). Few clusters are seen and  $\text{R-}\alpha_2\text{M}$  seems to be diffusely distributed on the cell surface. By titration it is possible to estimate roughly the concentration of inhibitor required to produce a 50% decrease in the number and intensity of fluorescent spots ( $\text{ID}_{50}$ ). Concentrations three- to fivefold below the  $\text{ID}_{50}$  produced no observable effect. Concentrations near the  $\text{ID}_{50}$  partially inhibited clustering (Fig. 2b), and concentrations three- to fivefold higher than the  $\text{ID}_{50}$  almost completely blocked clustering (Fig. 2c). In the case of dansylcadaverine, we estimated an  $\text{ID}_{50}$  of 20  $\mu\text{M}$ . The effect of dansylcadaverine was not unique to CHO cells. Equivalent concentrations of dansylcadaverine blocked clustering and internalisation of  $\text{R-}\alpha_2\text{M}$  in NRK cells and Swiss 3T3 fibroblasts (data not shown). Dansylcadaverine was also equally effective in inhibiting the clustering of rhodamine-conjugated EGF on NRK cells (Fig. 2e, f) and on Swiss 3T3 cells (data not shown).

To rule out an effect of dansylcadaverine on  $\text{R-}\alpha_2\text{M}$  binding, CHO cells were incubated for 30 min with dansylcadaverine and then for 15 min more with added  $\text{R-}\alpha_2\text{M}$ . Both unbound  $\text{R-}\alpha_2\text{M}$  and dansylcadaverine were then washed out and the fate of the diffusely bound  $\text{R-}\alpha_2\text{M}$  was followed (Fig. 2d). Within 30 min, bright fluorescent spots formed, indicating that ligand previously bound to receptor was competent to cluster and be internalised. Dansylcadaverine did not significantly affect the fluorescent intensity of cells incubated with  $\text{R-}\alpha_2\text{M}$  (Table 1). Furthermore, dansylcadaverine did not reduce the binding of  $^{125}\text{I}$ -EGF by cells (H. Haigler and I.H.P., in preparation).

### Correlation of inhibition of clustering with inhibition of transglutaminase

Having established conditions for assaying inhibition of transglutaminase and also inhibition of receptor-mediated endocytosis, we compared the potency of various compounds as inhibitors of the two processes. The results are plotted in Fig. 3. The data obtained with CHO cells are shown by solid circles;



**Fig. 2** Effect of dansylcadaverine on R- $\alpha_2$ M and EGF clustering by CHO and NRK cells. *a*, R- $\alpha_2$ M distribution at 37 °C. Fluorescent spots indicate clustered R- $\alpha_2$ M on the cell surface and internalised fluorescent endocytotic vesicles. Dashed line shows outline of individual cell. *b*, R- $\alpha_2$ M + 20  $\mu$ M dansylcadaverine; *c*, R- $\alpha_2$ M + 100  $\mu$ M dansylcadaverine; *d*, R- $\alpha_2$ M + 100  $\mu$ M dansylcadaverine followed by washout of unbound R- $\alpha_2$ M and dansylcadaverine; *e*, NRK cell + rhodamine-lactalbumin-EGF (R-lac-EGF); *f*, NRK + R-lac-EGF + 40  $\mu$ M dansylcadaverine. CHO cells were plated on Falcon tissue culture dishes and grown for 48 h to confluency in DMEM plus 10% heated calf serum. Dishes were rinsed three times with 37 °C DMEM, and preincubated for 30 min at 37 °C in DMEM with or without dansylcadaverine. After preincubation, 20  $\mu$ l of R- $\alpha_2$ M (6 mg ml<sup>-1</sup> in PBS) was added and the incubation continued for 30 min at 37 °C. Normal rat kidney cells (NRK) were treated in the same fashion except that R-lac-EGF<sup>13</sup> (100 ng ml<sup>-1</sup>) was added. After incubation the media were removed and the dishes rinsed three times with 37 °C DMEM and then fixed for 5 min in 2% formaldehyde in PBS at room temperature. Fixative was removed, the dishes rinsed twice with PBS and then fluorescence examined by epifluorescence illumination in a Zeiss RA microscope equipped with a RCA 1030H silicon intensifier television camera. The fluorescence image on the video monitor was directly photographed using a Polaroid camera. In panel *d*, after incubation with R- $\alpha_2$ M, dishes were rinsed four times with DMEM and then incubated for 30 min at 37 °C in DMEM before fixation. All magnifications  $\times 1,467$ .

compounds assayed in permeabilised NRK cells, shown by solid squares, will be discussed in the following section. For transglutaminase inhibitors with two components in the inhibition curve, the value of  $K_i$  for the high-affinity site was used.

The most potent inhibitor of CHO transglutaminase tested was dansylcadaverine, with a  $K_i$  for the high-affinity site of 2.5  $\mu$ M. As discussed above, 20  $\mu$ M dansylcadaverine produced a 50% inhibition of R- $\alpha_2$ M clustering (Fig. 3, no. 1). Methylamine was much less effective than dansylcadaverine in inhibiting transglutaminase, with a  $K_i$  for the high-affinity site of 0.2 mM; high concentrations of methylamine (10 mM) were required to inhibit clustering (Fig. 3, no. 2). Ethylamine was more potent than methylamine both in the transglutaminase assay and as an inhibitor of clustering ( $ID_{50}$  2 mM) (Fig. 3, no. 3). *n*-Propylamine, isopropylamine and *n*-hexylamine, all of which were equivalent to ethylamine as inhibitors of CHO

transglutaminase, were also equivalent in terms of their ability to block R- $\alpha_2$ M clustering and internalisation. Finally, both the novel inhibitors of transglutaminase, bacitracin and tolbutamide, inhibited R- $\alpha_2$ M clustering. Bacitracin (Fig. 3, no. 4) was quite a potent inhibitor, with an  $ID_{50}$  of approximately 350  $\mu$ M. Bacitracin was effective as an inhibitor of  $\alpha_2$ M clustering in intact CHO, NRK and Swiss 3T3 cells. The oral hypoglycaemic agent, tolbutamide, blocked  $\alpha_2$ M clustering. In line with its low affinity for transglutaminase, quite high concentrations of tolbutamide were required to inhibit clustering; the  $ID_{50}$  was 20 mM (Fig. 3, no. 5). There was a clear correlation between the activity of compounds as inhibitors of CHO transglutaminase and their ability to inhibit the aggregation and internalisation of  $\alpha_2$ M by CHO cells.

### Inhibition of clustering in permeabilised NRK cells

The studies of inhibitors of CHO transglutaminase demonstrated a quantitative correlation between inhibition of the enzyme and inhibition of clustering. However, the inhibitors tested were all compounds capable of diffusing into cells. To examine a wider range of transglutaminase inhibitors, we treated cells with 10% dimethyl sulphoxide (DMSO). In these conditions, cells became permeable to low molecular weight compounds (as judged by a loss of Trypan blue exclusion) but retained the capacity to bind and cluster R- $\alpha_2$ M. These studies were carried out with NRK cells because they adhered to the substratum well enough to permit permeabilisation without detachment of cells.

The requirement for permeabilisation is demonstrated by the studies of putrescine inhibition of clustering (Fig. 4*a-c*). Putrescine is a good substrate for NRK transglutaminase ( $K_m$  8.5 mM). However, it does not readily diffuse into cells<sup>23</sup>. Figure 4*a* shows R- $\alpha_2$ M aggregation in permeabilised cells; clusters of fluorescent ligand are seen. Figure 4*b, c* shows that, whereas in an intact cell even 100 mM putrescine does not block clustering, in a permeabilised cell, 20 mM putrescine is sufficient to block R- $\alpha_2$ M clustering.

The development of a permeabilised cell model for receptor-mediated endocytosis allowed us to assay peptide inhibitors of transglutaminase. Liver transglutaminase is inhibited by a variety of lysyl peptides<sup>15</sup> of which *N*-acetyl-L-lysine-*O*-methyl ester is one of the more effective ( $K_i$  0.5 mM). This ester also blocked R- $\alpha_2$ M clustering in permeabilised NRK cells, but it was ineffective in intact NRK cells. The correlation of the  $ID_{50}$  (4 mM) in NRK cells and the  $K_i$  is shown in Fig. 3, no. 7. Another transglutaminase substrate tested was the glutamyl peptide, carbobenzoxy-glutamyl-glycine. This inhibitor differed from other inhibitors tested as it competes for the glutamyl binding site on transglutaminase. The compound is a weak inhibitor of liver transglutaminase ( $K_i$  7.5 mM)<sup>14</sup> and also a weak inhibitor of R- $\alpha_2$ M clustering ( $ID_{50}$  2 mM). This relationship is shown in Fig. 3, no. 9.

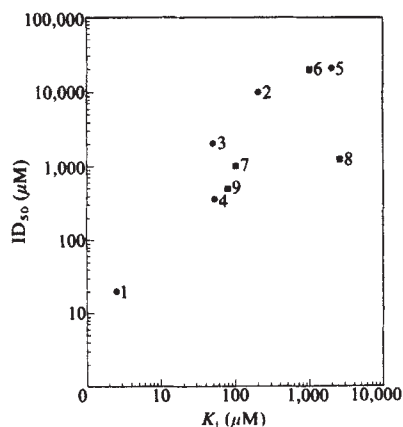
Another type of inhibitor tested was an irreversible site-specific inhibitor of transglutaminase, carbobenzoxy-diazonorvaline-paranitrophenyl ester (Z-DONVPA) (J. Folk, personal communication). Intact NRK cells were preincubated for 15 min at 37 °C with Z-DONVPA and then washed extensively

**Table 2** Effect of cell density on  $\alpha_2$ M clustering and transglutaminase activity in CHO cells

| Cell density                                      | Fluorescent clusters per cell<br>(mean $\pm$ s.d.) |                       |
|---------------------------------------------------|----------------------------------------------------|-----------------------|
|                                                   | Control                                            | +10 mM<br>methylamine |
| Low ( $0.2 \times 10^5$ cells cm <sup>-2</sup> )  | 9.9 $\pm$ 5.1                                      | <1                    |
| High ( $1.5 \times 10^5$ cells cm <sup>-2</sup> ) | 27.5 $\pm$ 11.2                                    | 6.9 $\pm$ 4.2         |

CHO cells were treated with R- $\alpha_2$ M (100  $\mu$ g ml<sup>-1</sup>) in conditions described in Fig. 1 legend. The total number of bright fluorescent spots associated with each was determined by focusing through the entire cell and counting the spots on a video monitor.





**Fig. 3** Correlation between inhibition of transglutaminase and inhibition of  $\alpha_2$ -M clustering. Compounds tested were: 1, dansylcadaverine; 2, methylamine; 3, ethylamine, *n*-propylamine, isopropylamine, *n*-hexylamine; 4, bacitracin; 5, tolbutamide; 6, putrescine; 7, *N*-acetyl-lysyl-*O*-methyl ester; 8, *N*-acetyl-(glycyl)<sub>2</sub>-L-leucyl-L-lysyl-(glycyl)<sub>2</sub>-glycine; 9, carbobenzoxy-glutamyl-glycine. ●, Compounds assayed for inhibition of clustering and inhibition of transglutaminase in CHO cells. The  $ID_{50}$  was estimated as the concentration required to produce a 50% decrease in the number and intensity of fluorescent spots and is reproducible within a factor of 2. The  $K_i$  was calculated from the formula

$$K_i = \frac{[I]_{0.5}}{1 + (S/K_i)}$$

where  $[I]_{0.5}$  was the concentration of inhibitor required to produce a 50% maximal inhibition of enzyme activity. The  $[I]_{0.5}$  was derived from the slope of the Eadie-Hofstee plots as shown in Fig. 1b, c. For dansylcadaverine and methylamine the  $[I]_{0.5}$  of the high-affinity component was used.  $S$  was the concentration of putrescine in the assay and  $K_i$  the Michaelis constant for putrescine; ■, compounds assayed for inhibition of clustering in permeabilised NRK cells (see Fig. 4 legend),  $K_i$  value reported for liver transglutaminase<sup>14,15</sup>. Linear regression analysis of the data gave a slope of 0.86, a  $y$  intercept of 1.3 and a correlation coefficient 0.83. The probability that this correlation was due to chance alone was  $<0.05$ .

before being exposed to R- $\alpha_2$ M. Z-DONVPA, 36  $\mu$ M, in the preincubation step produced complete and irreversible inhibition of R- $\alpha_2$ M clustering.

### Stereospecificity of inhibition of $\alpha_2$ M clustering

Folk and his colleagues have synthesised a heptapeptide model substrate of guinea pig liver transglutaminase, acetyl-(glycyl)<sub>2</sub>-L-leucyl-L-lysyl-(glycyl)<sub>2</sub>-glycine and have reported a  $K_i$  for this compound of 0.08 mM (ref. 15). As shown in Fig. 4d low concentrations of this peptide effectively inhibit R- $\alpha_2$ M clustering in permeabilised NRK cells. The  $ID_{50}$  for the heptapeptide was estimated to be 0.5 mM (Fig. 3, no. 8). Stereoisomers of the heptapeptide containing either D-lysine (L, D-heptapeptide) or D-leucine and D-lysine (D, D-heptapeptide) are much less active than the L-leucine, L-lysine (L, L-heptapeptide) derivative<sup>15</sup>. As shown in Fig. 4e, 1.5 mM D, D-heptapeptide produced no inhibition of R- $\alpha_2$ M clustering. At 8 mM, D, D-heptapeptide was roughly equivalent to 1.5 mM of the L, L derivative (Fig. 4f). The L, D-heptapeptide was comparable to the D, D stereoisomer (data not shown). Thus, the stereospecificity of the inhibition of  $\alpha_2$ M clustering matches the stereospecificity of guinea pig liver transglutaminase inhibition.

### Effects of cell density on R- $\alpha_2$ M clustering

During these studies, it was noted that CHO cells at low cell densities did not produce many fluorescent clusters when exposed to R- $\alpha_2$ M, whereas dense cultures produced many clusters. Birckbichler has reported that the transglutaminase activity of WI-38 fibroblasts rises at confluency<sup>24</sup>, and we have noted a precipitous rise in enzyme activity as CHO cells approach confluency. Table 2 compares the number of fluorescent clusters per cell in low cell density and high cell density CHO cells. Confluent cells cluster and internalise  $\alpha_2$ M

much more efficiently than low density cells. These cells also have much higher transglutaminase levels than low density cells<sup>25</sup>. We do not know if the increase in clustering is directly related to the increased transglutaminase activity, but it is consistent with that interpretation.

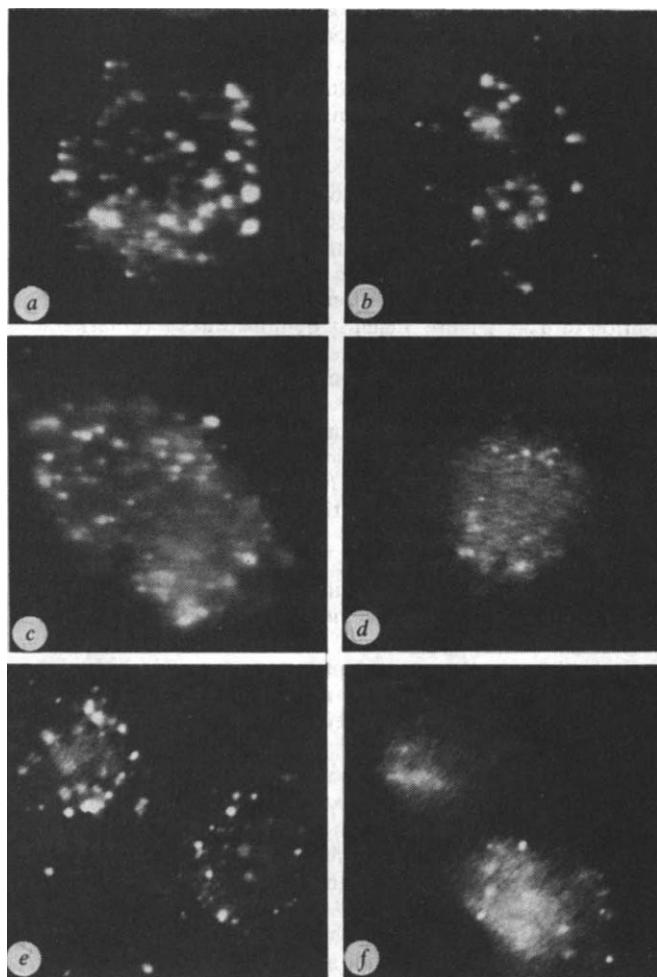
### Discussion

These studies were undertaken to test the hypothesis that a transglutaminase is involved in receptor-mediated endocytosis of polypeptide hormones and  $\alpha_2$ -macroglobulin. The approach was to see if a correlation could be made between the inhibition of transglutaminase and inhibition of the clustering and internalisation of  $\alpha_2$ M. Figure 3 clearly demonstrates a remarkable concordance between inhibitors of transglutaminase and inhibitors of  $\alpha_2$ -M clustering. The inhibitors range in potency over three orders of magnitude and include compounds as diverse as alkyl amines and diamines, lysyl peptides, a glutaminyl peptide, a peptide antibiotic and a sulphonylurea. Linear regression analysis of the data in Fig. 3 gives a slope to the regression line of 0.86, a correlation coefficient of 0.83 and a probability that the two variables are independently distributed of  $P < 0.05$ . The specificity of the correlation is clearly seen in the comparison of the stereospecificity of the two processes. A heptapeptide containing L-leucine and L-lysine inhibits both transglutaminase activity and R- $\alpha_2$ M clustering much more effectively than do the equivalent L, D or D, D stereoisomers. These observations indicate that it is very likely that a transglutaminase is required for the receptor-mediated endocytosis of certain ligands.

Several points have arisen from these studies. First, not all the transglutaminase activity in cell extracts is essential to hormone internalisation. Only the fraction of activity inhibited by low concentrations of dansylcadaverine, and by low concentrations of methylamine and other alkylamines seems to be related to receptor-mediated endocytosis. A major fraction of the transglutaminase activity in CHO cells was unaffected by most alkylamines, bacitracin or tolbutamide and could only be inhibited by high concentrations of dansylcadaverine or methylamine. These properties were unrelated to inhibition of  $\alpha_2$ M clustering. On the basis of its susceptibility to inhibition, we believe the 'essential' transglutaminase activity resembles liver transglutaminase. The other form of the enzyme may be a modified form of liver transglutaminase, a proenzyme or a completely different enzyme. Fractionation of the activities will be required to resolve this question.

A second point is that 5–10 times more inhibitor was required consistently to produce a 50% inhibition of  $\alpha_2$ M clustering than was required to produce a 50% inhibition of 'high-affinity' CHO transglutaminase. Several explanations may account for this discrepancy. It could be that 90% inhibition of ligand clustering and internalisation is only perceived as a 50% decrease in the number and intensity of fluorescent spots. Alternatively, 90% inhibition of enzyme activity may be required to interfere with ligand clustering at all. Another possible explanation is that the competition against endogenous substrates in the *in vivo* endocytosis assay may require higher concentrations of inhibitor than the competition against exogenous substrates in the *in vitro* transglutaminase assay. A fourth possibility, which we consider unlikely in view of the striking correlation in Fig. 3, is that receptor-mediated endocytosis requires an enzyme that is not transglutaminase but shares a very similar affinity for the wide variety of inhibitors tested. Although the discrepancies in  $ID_{50}$  and  $K_i$  values cannot be ignored, we do not think they refute the demonstrated relationship between transglutaminase and endocytosis.

Another point which has developed from these studies concerns the location of the transglutaminase activity essential for  $\alpha_2$ M aggregation. Inhibitors such as aliphatic amines or large aromatic molecules (such as dansylcadaverine) would be expected to diffuse readily into cells and these compounds were active in intact cells. Diamines and peptides, on the other hand, were active only after the cell had been permeabilised. This



**Fig. 4** Inhibition of R- $\alpha_2$ M clustering in permeabilised NRK cells, and effect of putrescine and stereoisomers of a lysyl peptide. *a*, R- $\alpha_2$ M clustering on permeabilised NRK cells; *b*, R- $\alpha_2$ M clustering in the presence of 100 mM putrescine on non-permeabilised NRK cells; *c*, inhibition of R- $\alpha_2$ M clustering by 20 mM putrescine on permeabilised NRK cells; *d*, inhibition of R- $\alpha_2$ M clustering by 1.5 mM acetyl-(glycyl)<sub>2</sub>-L-leucyl-L-lysyl-(glycyl)<sub>2</sub>-glycine (L,L-isomer) on permeabilised NRK cells; *e*, R- $\alpha_2$ M clustering in the presence of 1.5 mM Ac-(glycyl)<sub>2</sub>-D-leucyl-D-lysyl-(glycyl)<sub>2</sub>-glycine (D,D-isomer) on permeabilised NRK cells; *f*, R- $\alpha_2$ M clustering in the presence of 8 mM Ac-(glycyl)<sub>2</sub>-D-leucyl-D-lysyl-(glycyl)<sub>2</sub>-glycine (D,D-isomer) on permeabilised NRK cells. Dishes containing normal rat kidney fibroblasts (NRK cells) were rinsed twice with DMEM at 37 °C containing 10% DMSO and then incubated at 37 °C for 30 min in the same media supplemented with inhibitor. The dishes were then gently rinsed twice with warm (37 °C) DMEM and incubated at 37 °C for 20 min in DMEM containing R- $\alpha_2$ M (40  $\mu$ g ml<sup>-1</sup>) and the same concentration of inhibitor as in the preincubation. Dishes were then washed, fixed and observed under epifluorescence illumination as described in Fig. 2 legend. All magnifications  $\times 972$ .

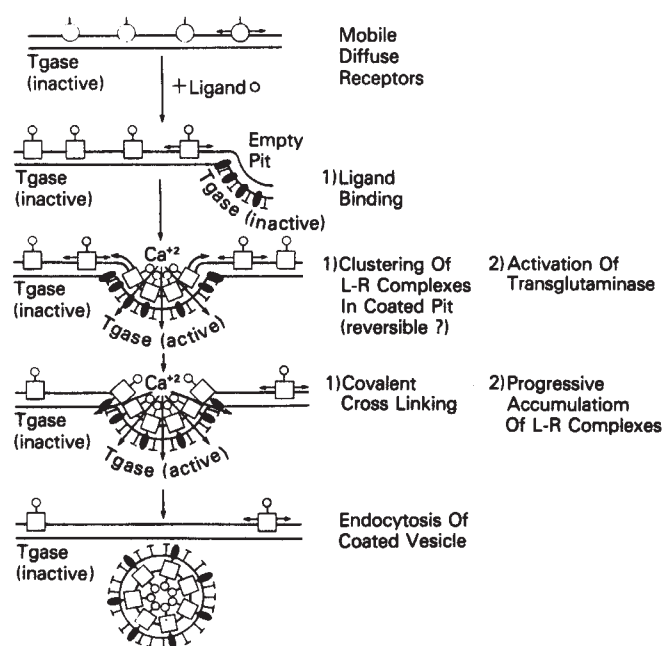
observation suggests that the active transglutaminase is inside the plasma membrane. Furthermore, localisation with anti-guinea pig liver transglutaminase antibodies in 3T3 cells have shown that a cross-reactive antigen (presumably transglutaminase) is located close to the inner aspect of the plasma membrane in the submembrane microfilament mat<sup>26</sup>. Therefore, it seems likely that aggregation of cell-surface receptors somehow involves activity of an enzyme located on the cytoplasmic side of the membrane.

Figure 5 presents a model for receptor-mediated endocytosis which includes a possible role for transglutaminase. Unoccupied receptors for hormones and  $\alpha_2$ M are diffusely distributed on the cell membrane. We suggest that in these conditions, transglutaminase in the cell is largely inactive because steady-state intracellular Ca<sup>2+</sup> levels are far below the levels required for enzyme activity (0.1–1 mM)<sup>27</sup>. Following binding of ligand,

occupied receptors may be altered so that they become associated in coated pits. We speculate that formation of small reversible aggregates in coated pits may promote local Ca<sup>2+</sup> influx and local activation of transglutaminase. Recently, Novogrodsky *et al.*<sup>28</sup> have reported that the binding of concanavalin A to lymphocytes rapidly activates transglutaminase. The presence of activated transglutaminase, elevated Ca<sup>2+</sup>, and aggregated ligand-receptor complexes might then result in covalent cross-linking of proteins in the complex. The proteins that might be cross-linked by such a reaction are not known. Receptors might be coupled to intrinsic membrane proteins or cytoskeletal proteins or coupled to each other. Recently, Baker *et al.*<sup>29</sup> and Linsley *et al.*<sup>30</sup> have reported covalent cross-linking of EGF, insulin and thrombin to their receptors during receptor-mediated endocytosis. The net effect of covalently cross-linking receptor proteins would be to make the aggregation process irreversible, and would result in a progressive accumulation of ligand-receptor complexes in the coated pit. Ultimately, the pit would pinch off and be internalised, resulting in the endocytosis of ligand-receptor complexes. The crucial step to confirming this hypothesis would be the identification of specific cross-linked proteins generated during receptor-mediated endocytosis.

This hypothetical scheme may not be applicable to all forms of receptor-mediated endocytosis or all routes of hormone internalisation. Some receptors, such as the LDL receptor, aggregate over coated pits even in the absence of ligand binding<sup>1</sup>. Protein cross-linking may not be required to stabilise these ligand-receptor complexes. In a human carcinoma cell line, A431, on the other hand, most EGF-receptor aggregates form over non-coated membrane and may be internalised by pinocytosis or by other mechanisms different from receptor-mediated endocytosis<sup>11</sup>. This form of hormone internalisation may not require transglutaminase activity. The biological significance of these different routes of endocytosis is not clear, but it is possible that cross-linking of proteins during internalisation may be of particular importance for receptor-mediated endocytosis that is coupled to receptor down-regulation.

In these studies, we have used pharmacological manipulation to alter some of the activities of transglutaminase in cells and have observed a concordant change in the ability to aggregate and internalise receptor-bound ligands. However, transglutaminase activity is under physiological control. As the data



**Fig. 5** Hypothetical model for receptor-mediated endocytosis of  $\alpha_2$ M and polypeptide hormones. The steps in the model are described in the text. Tgase, transglutaminase.



in Table 2 demonstrate, changes in cell density are correlated with changes in ligand clustering and endocytosis. Transglutaminase activity in cells varies with cell density<sup>24</sup>, with changes in intracellular cyclic AMP<sup>25</sup> and in response to viral transformation<sup>24</sup>. Such alterations in transglutaminase activity may be reflected in an alteration in the ability of cells to internalise surface-bound ligands. Maxfield *et al.*<sup>31</sup> have shown that blocking EGF internalisation with inhibitors of transglutaminase produced marked stimulation in the mitogenic

response of cells to the hormone. Changes in transglutaminase activity may therefore profoundly modify the way that cells respond to hormones.

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# Mapping of metabolites in whole animals by <sup>31</sup>P NMR using surface coils

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*The metabolic state of skeletal muscle and brain within intact rats is monitored using high resolution phosphorus nuclear magnetic resonance. Regional disturbances in metabolism (for example, localised ischaemia) are easily observed, indicating the diagnostic possibilities of the method. Measurements are made using 'surface' radio-frequency coils, which we discuss in detail.*

It was demonstrated in 1974 that phosphorus nuclear magnetic resonance (<sup>31</sup>P NMR) can be used to observe the major phosphorus-containing metabolites in intact, excised muscle<sup>1</sup>. Since then the method, which is non-destructive, has been used to study a variety of biochemical, physiological and medical problems in skeletal muscle<sup>2,3</sup>, heart<sup>4,5</sup>, kidney<sup>6</sup>, liver<sup>7</sup> and brain<sup>8</sup> (for reviews see refs 9–11). Apart from the measurements on mouse brain<sup>8</sup> and the recent experiments on heart in open-chested rats<sup>12,13</sup>, all these studies were done on excised organs, albeit often in a physiologically functional state.

Recently, 'spin-imaging' of water in living systems has demonstrated the ability of NMR to obtain spatial discrimination<sup>14</sup>. The combination of high resolution <sup>31</sup>P NMR with some method of spatial selection could offer a unique opportunity for the study of metabolism in defined tissues and organs of an intact animal. However, because of the low sensitivity and difficulties with spectral resolution, many of the methods used in water imaging are not yet practical for <sup>31</sup>P

spin-imaging of living systems. We describe here a simple method for detecting compounds in localised regions adjacent to the surface of the sample. We have used an unusual type of radiofrequency coil, which we call 'surface coil', to obtain the NMR signal. In this article we discuss the theory of the method, describe experiments on test phantoms, and show the application of the technique to the study of muscle and brain metabolism in living animals.

## Theory and design of surface coils

The design of radiofrequency (r.f.) coils is best considered in terms of the theory of Hoult and Richards<sup>15</sup>. Let us consider a single r.f. coil that serves the role of both transmitter and receiver. Their theory shows that:

- (1) The signal-to-noise ratio resulting from a 90° r.f. pulse applied to a small sample at point *q* is proportional to the value of  $(B_1)_{xy}$  at that point.  $(B_1)_{xy}$  is the component of the r.f. magnetic field in the *xy* plane (that is, in the plane perpendicular to the static field  $B_0$ ) that is generated by unit current passing through the coil.
- (2) The 90° pulse length for a sample at any point *q* is inversely proportional to the value of  $(B_1)_{xy}$  at that point.
- (3) The signal-to-noise ratio resulting from a single r.f. pulse of angle  $\phi$  is proportional to  $(B_1)_{xy} \sin \phi$ .  $\phi$  is equal to  $\gamma(B_1)_{xy} \tau$  where  $\tau$  is the length of the pulse,  $\gamma$  is the magnetogyric ratio of the nucleus, and *i* is the current passing through the coil.

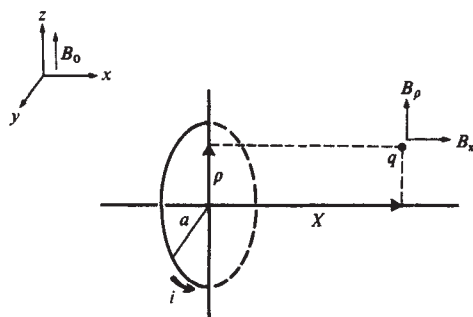
Clearly the field distribution  $(B_1)_{xy}$  produced by the coil is a critical factor in its performance. Conventional saddle-shaped and solenoidal coils are generally designed to produce a homogeneous  $B_1$  field throughout the sample volume; this ensures that all regions of a uniform sample contribute equally to the signal. In contrast, we show that a different coil design enables us to observe signals from a selected region adjacent to the surface of the sample.

Consider a single-turn flat circular coil orientated as in Fig. 1. The magnetic field generated at any point  $q$  by unit current flowing in the coil is given by Smythe<sup>16</sup> and is

$$B_\rho = \frac{\mu}{2\pi} \frac{x}{\rho[(a+\rho)^2 + x^2]^{1/2}} \left[ -K + \frac{a^2 + \rho^2 + x^2}{(a-\rho)^2 + x^2} E \right] \quad (1)$$

$$B_x = \frac{\mu}{2\pi} \frac{1}{[(a+\rho)^2 + x^2]^{1/2}} \left[ K + \frac{a^2 - \rho^2 - x^2}{(a-\rho)^2 + x^2} E \right] \quad (2)$$

$B_x$  and  $B_\rho$  are the axial ( $x$ ) and radial ( $\rho$ ) components of the field at point  $q$ ,  $a$  is the coil radius,  $\mu$  the permeability of the medium about the coil and  $K$  and  $E$  are complete elliptic integrals of the first and second kind.



**Fig. 1** Diagram illustrating the orientation of a single loop coil. Various parameters are  $B_0$ , the main magnetic field,  $a$ , the coil radius,  $i$ , the current flowing in the coil, and  $q$ , a point in space described by the coordinates  $(\rho, x)$ .  $B_\rho$  and  $B_x$  are the  $B_1$  field components at  $q$  resulting from current  $i$ . The angle  $\theta$  formed by the vector  $\rho$  and  $B_0$  is shown as 0 for clarity of presentation.

From Fig. 1, we see that the direction of  $B_x$  is always perpendicular to that of the static field  $B_0$ , while the perpendicular component of  $B_\rho$  [ $(B_\rho)_{xy}$ ] is given by

$$(B_\rho)_{xy} = B_\rho \sin \theta \quad (3)$$

where  $\theta$  is the angle between the directions of  $B_\rho$  and  $B_0$  (see Fig. 1 where  $\theta = 0$  for clarity of presentation). The magnetic field  $(B_1)_{xy}$  generated at any point  $q$  therefore depends on  $\theta$  and is given by

$$(\overline{B_1})_{xy} = \overline{B_x} + (\overline{B_\rho})_{xy} \quad (4)$$

A plot of  $(B_1)_{xy}$  as a function of  $\rho$  derived from equations (1)–(4) is given in Fig. 2 for the two extremes  $\theta = 0$  and  $\theta = \pi/2$ .

Following from the relationships given above (points (1) to (3)), it is possible to map the  $(B_1)_{xy}$  field of any r.f. coil. We have mapped  $(B_1)_{xy}$  for a single-turn flat circular coil of radius 0.75 cm by placing a small sample (a disk of volume 0.01 ml containing 12 M phosphoric acid and 4 mM  $\text{NiCl}_2$ ) at different positions  $q$  relative to the coil. The field  $(B_1)_{xy}$  as determined

from the variation in signal intensity is plotted in Fig. 2 and, as can be seen, shows good agreement with theory. Similar agreement was obtained from the variation in  $90^\circ$  pulse length, and from experiments with other surface coils. Note that the  $^{31}\text{P}$  test sample was surrounded by an aqueous medium of 154 mM NaCl to approximate the electrical characteristics of animal tissue.

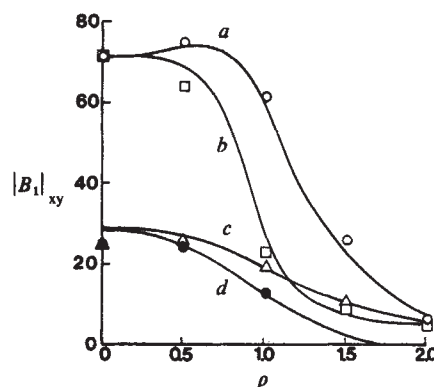
$(B_1)_{xy}$  is localised in a volume about the centre of the coil roughly defined by the coil radius (Fig. 2). It should therefore be possible to observe high resolution NMR signals from selected regions adjacent to the surface of the samples. Furthermore, we should stress that the region of sample contributing significant signal intensity will be spatially far more sharply defined than  $(B_1)_{xy}$  due to the sine term in point (3) above.

Finally, as Hoult and Richards<sup>15</sup> have suggested, variations in phase of  $(B_1)_{xy}$  should not affect this type of single coil experiment. This is because phase changes induced at transmission are mirrored at reception, and thus cancel. This conclusion was confirmed by our experiments.

## Experiments with phantoms

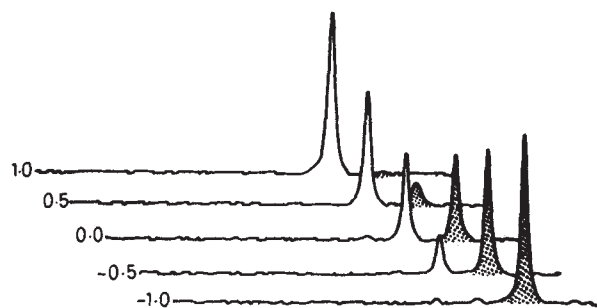
To verify and more clearly illustrate the spatial resolution of surface coils,  $^{31}\text{P}$  NMR experiments were performed on two different phantom samples. The first sample consisted of a two compartment rectangular box ( $10 \times 2 \times 1$  cm) partitioned by a thin panel halfway along the length (5 cm) and filled with a solution of  $\text{Na}_2\text{HPO}_4$  (100 mM) in one compartment and phosphocreatine (100 mM) in the other.  $^{31}\text{P}$  NMR spectra were collected using a flat four-turn surface coil (mean radius 0.55 cm) placed outside the box at various positions along its length. The results are shown in Fig. 3. With the coil placed directly over the dividing partition, position 0.0, both compartments contribute equal signal intensity. When the centre of the coil is one radius from either side of the partition, only that compartment directly beneath the coil gives a signal. Thus sample selectivity (as a function of the radial,  $\rho$ , coordinate) is approximately confined to an area bounded by the coil circumference.

The second phantom consisted of a box ( $4 \times 3 \times 3$  cm) filled with an aqueous solution of  $\text{NaH}_2\text{PO}_4$  (20 mM) and NaCl (100 mM). The same coil was placed on the outside of the box (wall thickness 0.1 cm) and the  $^{31}\text{P}$  spectrum obtained. A hollow



**Fig. 2** The spatial dependence of  $(B_1)_{xy}$  as a function of  $\rho$ , the radial coordinate.  $\rho$  is expressed in units of radius,  $a$ , while  $(B_1)_{xy}$  is normalised to 100 at the centre of the coil,  $(\rho, x) = (0, 0)$ . The solid curves were derived from equations (1)–(4) while the symbols represent experimental data. Spatial parameters vary for each curve as follows: curve  $a$ ,  $\theta = \pi/2$  and  $x \approx 0.5a$ ; curve  $b$ ,  $\theta = 0$  and  $x \approx 0.5a$ ; curve  $c$ ,  $\theta = \pi/2$  and  $x = 1.1a$ ; curve  $d$ ,  $\theta = 0$  and  $x = 1.1a$ .





**Fig. 3** Stacked plot demonstrating the radial,  $\rho$ , resolution of surface coils. The two  $^{31}\text{P}$  resonances, each arising from a separate compartment in the phantom, are phosphocreatine (shaded) and inorganic phosphate ( $\text{Na}_2\text{HPO}_4$ ) (non-shaded). The label associated with each spectrum is the distance (in units of coil radius,  $a$ ) between the surface coil centre and the partition which separates the two compartments.

glass disk of radius 0.75 cm and thickness 0.5 cm filled with a solution of NaCl (154 mM) was then placed in the solution-filled box, immediately in front of the coil. On repeating the measurement, a sixfold reduction in signal intensity was observed. This results from the exclusion of 0.8 ml of the  $\text{NaH}_2\text{PO}_4$  solution in front of the coil, illustrating the three-dimensional spatial selectivity of this type of NMR coil.

These experiments verify the theoretical predictions (Fig. 2) and the properties of these coils can be summarised as follows: (1) The high resolution NMR experiment can be performed with a surface coil external to the sample; (2) the spatial resolution is well defined and can be theoretically and experimentally determined; (3) this spatial resolution may be adjusted by appropriate choice of coil radius and pulse width; (4) the filling factors of surface coils compare favourably with those of conventional coils; and (5) noise generated by r.f. losses in conducting samples<sup>17</sup> arise only from those regions of the samples that contribute signal. These latter two points suggest that good signal-to-noise ratios can be expected with well-designed surface coils.

## Measurements of metabolites in whole animals

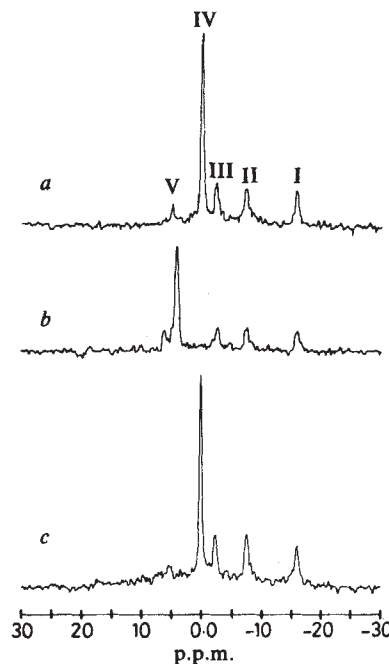
The results and calculations presented above, together with studies on tissues and perfused organs provided the rationale for attempting to observe  $^{31}\text{P}$  NMR spectra from muscle and brain of an intact rat.

Figure 4a shows the  $^{31}\text{P}$  NMR spectrum obtained by placing the four-turn surface coil against the leg of an anaesthetised rat. The resonances arise from the three phosphates of ATP, phosphocreatine and inorganic phosphate ( $\text{P}_i$ ), within the leg muscle.  $^{31}\text{P}$  signals contributed by blood within the muscle tissue are negligible as fresh blood contains only a small amount of ATP and no phosphocreatine relative to 2,3-diphosphoglycerate<sup>18</sup>. (If 2,3-diphosphoglycerate were present in significant quantities, it would produce two signals with chemical shifts of about 5 and 6.5 p.p.m.)

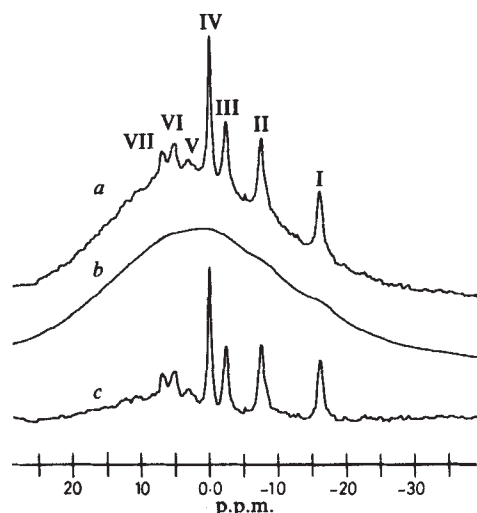
To achieve conditions where peak areas are directly proportional to the amounts of tissue metabolites, a 12-s delay was used between sampling pulses. (This follows from the  $^{31}\text{P}$   $T_1$  relaxation times for intracellular  $\beta$ -ATP (~1 s), phosphocreatine (~3 s) and inorganic phosphate (~3 s).) Thus the spectral accumulation of 128 scans required about 25 min. However, by reducing the delay between pulses, satisfactory spectra can be recorded in less than a minute. These can be used to follow changes in metabolites, to compare different metabolic states, or even to perform quantitative studies if the appropriate relaxation times or saturation factors are accurately determined<sup>10</sup>.

A measure of the ATP concentration is obtained from the area of the signal at -16 p.p.m. (the  $\beta$ -phosphate peak of ATP). The resonance at -2.4 p.p.m. contains contributions from the  $\gamma$ -phosphate of ATP and the  $\beta$ -phosphate of mobile ADP. As the ratio of the areas of these two peaks ( $[\text{ATP}]/[\text{ADP}] + [\text{ATP}]$ ) is one, within experimental error ( $0.94$ ,  $n = 5$ , s.e.m.  $\pm 0.05$ ) clearly the concentration of mobile ADP is too low to be observed. The phosphocreatine to ATP ratio is  $4.04 \pm 0.18$  ( $n = 5$ ) which compares reasonably with recent freeze clamping values for rat skeletal muscle<sup>19</sup> but is considerably higher than other reported values<sup>20</sup>. Because phosphocreatine is rapidly degraded during ischaemia<sup>1</sup> we suggest that our *in vivo* ratio of phosphocreatine to ATP represents the best estimate so far for this parameter that reflects the energy status of the cell. The measurement of inorganic phosphate by freeze extraction procedures provides special difficulties, and it is significant that the NMR results give a phosphocreatine:  $\text{P}_i$  ratio of  $14.6$  ( $n = 5$ ; s.e.m.  $\pm 1.20$ ). Assuming a phosphocreatine concentration of  $23.1 \mu\text{mol per g wet wt}$  (ref. 19), the  $\text{P}_i$  concentration derived from NMR is  $1.6 \mu\text{mol per g wet wt}$ , a value considerably lower than that derived from freeze clamping techniques<sup>20</sup>. This observation might explain why the activity of phosphorylase *b* in resting muscle is considerably less than that predicted by studies on the isolated enzyme combined with metabolic measurements by freeze extraction<sup>21</sup>.

Following the observations of Moon and Richards<sup>22</sup> it has been well established that the position of the inorganic phosphate resonance provides a good measure of intracellular pH



**Fig. 4**  $^{31}\text{P}$  NMR spectra of the leg muscle of an intact rat obtained with a surface coil. Each spectrum represents 128 scans over 25 min. A 15-Hz line-broadening exponential multiplication was used to enhance signal-to-noise. *a*, Non-ischaemic muscle below the knee joint; *b*, ischaemic muscle from the same area as in *a* after application of a tourniquet just above the knee; *c*, spectrum of muscle above the tourniquet. Chemical shift assignments for non-ischaemic rat leg muscle are  $\beta$ -phosphate of ATP (I), -16.10 ( $n = 5$ , s.e.m.  $\pm 0.02$ ),  $\alpha$ -phosphate (II), -7.54 ( $n = 5$ ,  $\pm 0.00$ ),  $\gamma$ -phosphate of ATP (III), -2.47 ( $n = 5$ ,  $\pm 0.02$ ), phosphocreatine (IV), 0.00 by definition, and inorganic phosphate (V), 4.90 ( $n = 5$ ,  $\pm 0.03$ ). Chemical shifts are defined as positive in the high frequency direction and phosphocreatine is taken as an internal reference. Normally fed, pentobarbital anaesthetised male Wistar rats were used for all experiments. The rats were examined while in a vertical position. All experiments were done at 73.8 MHz on a spectrometer equipped with a wide-bore magnet.



**Fig. 5**  $^{31}\text{P}$  NMR spectra of the brain of an intact rat obtained with a surface coil. Spectra *a-c* demonstrate the effects of the convolution difference technique in eliminating the broad  $^{31}\text{P}$  component arising from bone. *a*, Original brain spectrum, 300 data accumulations over 60 min with a 15-Hz line broadening exponential multiplication; *b*, same as *a* except a 400-Hz line broadening exponential multiplication was used; *c*, difference spectrum of *a* minus *b*. Chemical shift assignments are  $\beta$ -phosphate of ATP (I),  $-16.20$  ( $n = 5$ ,  $\pm 0.05$ ),  $\alpha$ -phosphate of ATP (II),  $-7.62$  ( $n = 5$ ,  $\pm 0.02$ ),  $\gamma$ -phosphate of ATP (III),  $-2.48$  ( $n = 5$ ,  $\pm 0.02$ ), phosphocreatine (IV)  $0.00$  by definition, phosphodiester (V)  $3.0$  ( $n = 3$ ,  $\pm 0.1$ ), inorganic phosphate and the 2-phosphate of 2,3-diphosphoglycerate (VI),  $5.00$  ( $n = 5$ ,  $\pm 0.007$ ), and sugar phosphates and the 3-phosphate of 2,3-diphosphoglycerate (VII),  $6.65$  ( $n = 5$ ,  $\pm 0.06$ ).

(refs 9, 10). The chemical shift of  $\text{P}_i$  within rat leg muscle is  $4.90 \pm 0.03$  ( $n = 5$ ), consistent with a  $\text{pH}$  of  $7.1 \pm 0.02$  ( $n = 5$ ).

In an experiment similar in philosophy to the two compartment phantom, the effect of localised ischaemia in the rat leg muscle was assessed (Fig. 4). A control muscle spectrum was obtained from just below the knee joint (Fig. 4*a*). Then a very tight tourniquet was placed around the leg just above the knee and a spectrum obtained from the same location as the initial control (Fig. 4*b*). The two spectra are significantly different. The ischaemic region of the muscle has no phosphocreatine, somewhat reduced amounts of ATP and a very large  $\text{P}_i$  concentration. In addition, the  $\text{P}_i$  chemical shift ( $4.31$  p.p.m.) differs significantly from that in the control muscle ( $4.92$  p.p.m.), indicating a more acidic  $\text{pH}$  of  $6.7$ . (Similar results with excised tissue have been observed<sup>1-3</sup>.) The small new peak at  $6.30$  p.p.m. may be attributable to the build-up of sugar phosphates or nucleotide monophosphate. Finally, with the tourniquet still in place, a spectrum was obtained from the leg muscle

above the tourniquet (Fig. 4*c*). This spectrum is very similar to that of the first control, indicating healthy, non-ischaemic muscle. The localisation of ischaemic areas in diseased muscle is thus clearly possible.

One of the most difficult organs to study by biochemical and perfusion techniques is the brain. Because of the rapid oxidative metabolism and sensitivity to short periods of hypoxia or ischaemia of the brain, considerable effort has been spent in developing a variety of techniques such as 'freeze-blowing'<sup>23</sup>, and cryo-NMR<sup>8</sup> to assess the *in vivo* energy status of this tissue.

By placing a surface coil on top of the rat's head, directly over the brain, we were able to obtain excellent  $^{31}\text{P}$  spectra from the brain (Fig. 5). As there is no muscle tissue between the coil and the brain these experiments are selective for brain tissue and bone only. The very broad contribution from the bone can easily be eliminated by the convolution difference technique<sup>24</sup>.

From the chemical shifts of the three ATP resonances in brain and muscle we can conclude that in both tissues the ATP is predominantly complexed to divalent cations which we assume to be  $\text{Mg}^{2+}$ .

The energy status of the live brain may be best compared with that of perfused or rapidly frozen tissue from the value of the phosphocreatine to ATP ratio. The value we derived from NMR ( $1.93 \pm 0.12$ ,  $n = 5$ ) is higher than the best values obtained by rapid freezing<sup>23</sup>. In addition we estimate that the concentrations of both mobile ADP and  $\text{P}_i$  are much lower than those measured by destructive analytical techniques. In the brain spectrum the ratio of the resonances  $\beta$ -phosphate of ATP:  $\gamma$ -phosphate of ATP +  $\beta$ -phosphate of ADP is  $1.07 \pm 0.08$  ( $n = 5$ ) indicating that ADP is not detectable. The  $\text{P}_i$  signal is obscured by the 2-phosphate peak of 2,3-diphosphoglycerate present in blood. The 3-phosphate from the same compound is at  $6.6$  p.p.m. and indicates that the  $\text{P}_i$  concentration in the brain must be well below that of ATP, in contrast to values obtained from freeze extraction. These considerations lead to the conclusion that the phosphorylation state in brain tissue ( $[\text{ATP}]/[\text{ADP}][\text{HPO}_4^{2-}]$ ) is at least an order of magnitude higher than the generally accepted value of  $\sim 3,400 \text{ M}^{-1}$ .

## Conclusions

The use of surface coils to obtain high resolution NMR spectra from selected regions of large objects is clearly not limited to the systems described here. The design of the coil can be adapted to suit the sample. In animals, tissues other than muscle and brain (for example, fat tissue or cancerous growth) could be studied. Coupled with surgery, internal organs can easily be investigated<sup>12</sup>.

An early low resolution application of flat coils has been for the measurement of blood flow<sup>25</sup>. Plant, fungal or non-living materials can also be examined in this way.

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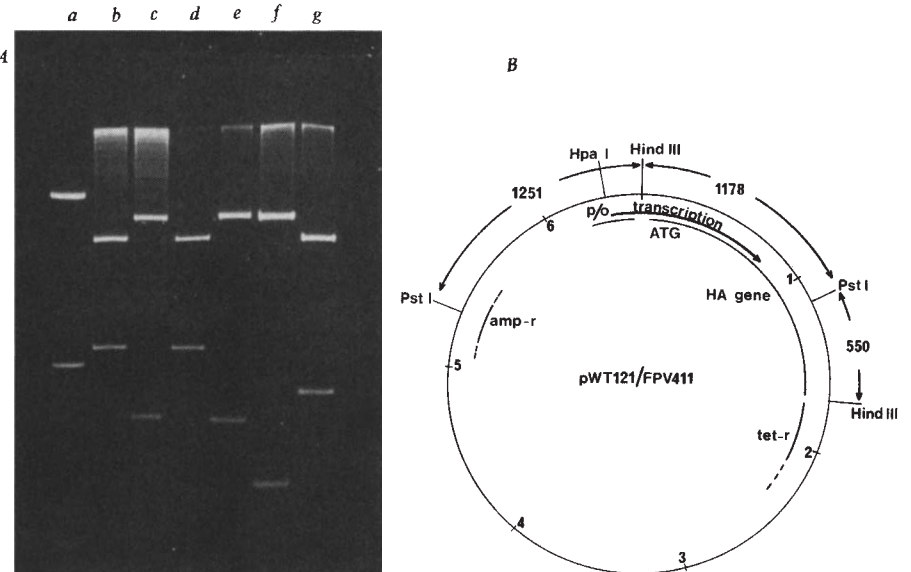
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**Fig. 1 A.** Predicted nucleotide sequences around the *Hind*III sites of pWT111, pWT121 and the FPV-HA gene. Arrows indicate the position of the *Hind*III sites and, for clarity, only the sequence of the coding strand is shown. The protein sequence of the N-terminus of anthranilate synthetase has previously been reported by Lee *et al*<sup>10</sup>. The numbering system is bidirectional beginning with the 5'-nucleotide of the gene 4 complementary RNA. B, Potential base pairing between the 3' oligonucleotide of *E. coli* 16S RNA<sup>11</sup> and the pre-AUG region of the haemagglutinin mRNA.

**Fig. 2 A**, Orientation of the FPV gene in pWT111, pWT121 and pBR322. pBR322, pWT111 or pWT121 (10  $\mu$ g) was limit digested with *Hind*III. The resulting 5'-terminal phosphates of the vectors were removed by treatment with 20  $\mu$ g bacterial alkaline phosphatase for 30 min at 37 °C in a 25  $\mu$ l incubation containing 20 mM Tris-HCl pH 7.5 and 0.1% SDS. After phenol extraction and ethanol precipitation 0.2  $\mu$ g of each DNA was ligated to ~40 ng FPV-HA gene in a 20  $\mu$ l incubation containing 50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 20 mM dithiothreitol, 1 mM ATP and 0.02 units T4 DNA ligase (New England Biolabs). After overnight incubation at 15 °C the mixtures were diluted to 100  $\mu$ l with TCM (10 mM Tris-HCl pH 7.5, 10 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>) and used to transform 200  $\mu$ l CaCl<sub>2</sub>-treated *E. coli* K12 HB101 by a previously described procedure<sup>12</sup>. Transformants present after growth for 16 h at 37 °C on L-agar<sup>13</sup>, plus 100  $\mu$ g ml<sup>-1</sup> carbenicillin (Pyopen) were tested for tetracycline resistance by picking onto agar plates containing M9 salts, glucose and casamino acids<sup>13</sup> plus carbenicillin and tetracycline (10  $\mu$ g ml<sup>-1</sup>). For plasmid isolation colonies were picked and 100-ml cultures grown in M9 salts, glucose, casamino acids medium supplemented with carbenicillin. At an A<sub>600</sub> of ~0.6, chloramphenicol was added to a final concentration of 150  $\mu$ g ml<sup>-1</sup> and incubation continued for 16 h at 37 °C. Cells were killed by the addition of diethylpyrocarbonate<sup>14</sup> to 0.4%, collected by centrifugation, washed with TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and suspended in 1.5 ml of iced 25% sucrose in 50 mM Tris-HCl pH 8. Cells were lysed by the addition of 0.5 ml lysozyme (10 mg ml<sup>-1</sup>, 0.5 M, pH 8) and 2.5 ml iced Triton solution (0.1% Triton X-100, 50 mM Tris-HCl pH 8, 50 mM EDTA) at 5-min intervals. The resulting lysates were cleared by spinning at 15,000g for 20 min, extracted with phenol and then chloroform and finally precipitated by the addition of 0.1 vol 3M NaAc and 0.54 vol isopropanol. After 30 min at -20 °C the DNA was pelleted, washed with 70% ethanol, dried and dissolved in TE. *Pst*I digests were carried out on 0.1  $\mu$ g DNA in conditions recommended by the vendor (New England Biolabs) with the modification that RNase (50  $\mu$ g ml<sup>-1</sup>) was included in the incubation. Digests were fractionated on a 1.4% agarose slab gel<sup>15</sup> and bands visualised under UV light after staining with ethidium bromide. Lane a contains PM-2 DNA digested with *Hind*III. Bands seen are 5,400, 2,350 and 1,050 base pairs. The other lanes contain *Pst*I digests of the following plasmids: b, pWT121/FPV 411(R); c, pWT121/FPV 412(L); d, pWT111/FPV 502(R); e, pWT111/FPV 503(L); f, pBR322/FPV 604(L); g, pBR322/FPV 605(R). **B**, Structure of pWT121/FPV 411(R). pWT121 contains a single *Hind*III site downstream from the *trp* promoter<sup>9</sup>. The plasmid shown contains the FPV-HA gene cloned at the *Hind*III site in the R orientation. The distances between the *Pst*I and *Hind*III sites are shown as well as the direction of transcription from the *trp* promoter and the position of the initiator ATG. A full description of the construction of the pWT plasmid series has been published elsewhere<sup>9</sup>. Briefly, the 497-base pair *Hinf*I fragment containing the *trp* promoter/operator region as well as the nucleotides specifying the leader sequence and first seven amino acids to *trpE* (ref. 10) was isolated and cloned, using *Hind*III linkers, into the *Hind*III site of pBR322. From this a plasmid, pWT101, was isolated containing the *trp* promoter in the R orientation. In pWT101 tetracycline resistance is controlled from the *trp* promoter. Elimination of the *Hind*III site upstream of the *trp* promoter produced the vector pWT111 and phase changing at the *Hind*III site of pWT111 using DNA polymerase I and *Hind*III linkers produced pWT121 and pWT131.



production. That is, a gene in the R orientation required rightward (clockwise) transcription for expression; in the pWT plasmid series this is the orientation for expression from the *trp* promoter. For clarity we have included the letter (R) or (L) after the plasmid number to indicate its orientation. pWT121 contains a single *Pst*I site, in the ampicillin gene<sup>16</sup>, 1,251 base pairs from the *Hind*III site and the FPV HA gene contains a *Pst*I site 1,178 base pairs from the 5' end of the coding strand<sup>6</sup>. Thus *Pst*I digests of the recombinant plasmids should indicate which are correctly orientated with respect to the *trp* promoter. Representatives of the two FPV-gene containing groups are shown in Fig. 2A (b and c). The Tc<sup>S</sup> plasmids gave rise to a band of 1,800 base pairs on *Pst*I digestion while the Tc<sup>R</sup> plasmids produced one of 2,450 base pairs. These are consistent with the fragments expected from the plasmid shown in Fig. 2B and indicate that all the Tc<sup>S</sup> plasmids contained inserts in the L orientation while the Tc<sup>R</sup> plasmids contained inserts in the R orientation.

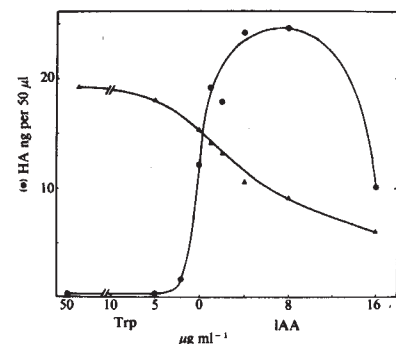
In a similar way we re-cloned the HA gene into the *Hind*III site of pWT111 and pBR322. Again the pWT111/FPV plasmids could be divided into Tc<sup>R</sup> and Tc<sup>S</sup> groups and *Pst*I restrictions (Fig. 2A, d and e) of representatives of these groups indicated that the HA gene was in the R orientation in the Tc<sup>R</sup> group and in the L orientation in the Tc<sup>S</sup> group. The pBR322/FPV plasmids however were all Tc<sup>S</sup> but still contained L and R orientated genes as shown in Fig. 2A (f and g).

The correct phasing of the HA gene in pWT121/FPV 411(R) with the *trpE* AUG was confirmed by nucleotide sequencing. The HA gene contains an *Eco*RII restriction site at positions 36–40 (ref. 6). This site, however, is methylated and although not cleaved by *Eco*RII, it is cleaved by the *Eco*RII isoschizomer *Bst*NI. Therefore we digested pWT121/FPV 411(R) with *Bst*NI, labelled the 5' ends with <sup>32</sup>P, recut the DNA with *Hpa*I and isolated the fragment containing the initiation region. The nucleotide sequence of this fragment was complementary to the sequence predicted in Fig. 1A, except for nucleotide -36 which

was an A instead of a G. This was also the case for the corresponding position in pWT111. In both plasmids this position corresponds to the 3'-terminal nucleotide of the *Hind*III linker. The reason for the change is not clear; it might be due to random mutation or, more likely, to micro-heterogeneity or incomplete deblocking after chemical synthesis at the 3' ends of the *Hind*III linker. In either event the effect in the mRNA strand is to have a serine codon (UCG) instead of a proline codon (CCG) but does not change the phase with respect to the *trpE* AUG.

## Gene expression

*E. coli* colonies containing representatives of the plasmids described above were screened for FPV-HA antigen using a solid-phase immunological method<sup>17</sup>. Briefly, small cultures of individual colonies were grown, collected and lysed using lysozyme and Triton X-100. Any HA sequences present were



**Fig. 3** Control of HA production. Overnight cultures of *E. coli* K12 HB 101 containing pWT121/FPV 411(R) were grown in M9 salts, glucose, casamino acids medium supplemented with carbenicillin (100  $\mu$ g ml<sup>-1</sup>). Aliquots of 100  $\mu$ l were inoculated into 5 ml of the above medium containing either tryptophan or IAA as indicated. Cultures were grown for 2 h at 37 °C, the A<sub>600</sub> determined and cells collected, lysed and assayed for HA content as described in Table 1.



**Table 1** Radioimmunoassay of FPV-HA in lysates of bacteria containing influenza genes

| Plasmid        | Orientation of gene | Phenotype       | HA content (ng per 50 µl) |
|----------------|---------------------|-----------------|---------------------------|
| pWT121/FPV 411 | R                   | Tc <sup>R</sup> | 20.3                      |
| pWT121/FPV 412 | L                   | Tc <sup>S</sup> | 5.0                       |
| pWT111/FPV 502 | R                   | Tc <sup>R</sup> | 4.1                       |
| pWT111/FPV 503 | L                   | Tc <sup>S</sup> | 2.1                       |
| pBR322/FPV 604 | L                   | Tc <sup>S</sup> | 3.6                       |
| pBR322/FPV 605 | R                   | Tc <sup>S</sup> | 0                         |
| pWT121         | —                   | Tc <sup>R</sup> | 0                         |
| pBR322         | —                   | Tc <sup>R</sup> | 0                         |

Liquid cultures (5 ml) of individual colonies were grown in M9 medium. Cells were collected by centrifugation, and lysed, in a final volume of 1 ml, as described in Fig. 2A. This lysate was used for antigen assay without further treatment. HA-antigen assays were performed in polystyrene culture tubes (Nunc no. 1410) using an antibody sandwich technique<sup>17</sup>. Rabbit anti-FPV HA was purified by ammonium sulphate precipitation and DEAE-cellulose chromatography<sup>18</sup> before use and labelled with <sup>125</sup>I as described elsewhere<sup>19</sup>. Tubes were coated with 50 µl of 0.2 M NaHCO<sub>3</sub> pH 9 containing 60 µg ml<sup>-1</sup> purified, unlabelled IgG for 10 min at room temperature, washed with 3 × 1-ml aliquots of wash buffer (phosphate-buffered saline containing 0.1% bovine serum albumin, 0.1% NP-40 and 0.5% normal goat serum) and then incubated at room temperature either with known amounts (1–20 ng) of Sarkosyl-disrupted FPV or with the bacterial lysates. After 2 h, the tubes were washed with 4 × 1-ml aliquots of wash buffer and finally incubated at 4°C for 16 h with 50 µl wash buffer containing 200,000 c.p.m. <sup>125</sup>I-anti-HA. Tubes were again washed and counted in a Packard γ-counter. Antigen present was quantitated from standard curves relating the radioactivity bound to the amount of FPV present.

bound to a polystyrene tube coated with FPV-HA specific IgG. Bound antigen was then detected by incubating with high specific activity <sup>125</sup>I-anti FPV-HA. As indicated in Table 1, immune reactivity was detected from all colonies containing an FPV-HA gene inserted into the pWT plasmids and from pBR322 containing the HA gene in the L orientation. Neither of the parent plasmids nor pBR322 containing the HA gene in the R orientation produced any immune reacting material. Further, the positive reactions were abolished if the tubes were coated either with normal rabbit IgG or with anti A/Victoria—HA IgG instead of specific anti-FPV-HA IgG.

It was interesting to find expression of HA antigen from one orientation of pBR322/FPV. The *Hind*III site of pBR322 lies in the promoter region of the tetracycline gene and it is known that cloning at the *Hind*III site destroys this rightward transcribing promoter<sup>20</sup> unless the cloned DNA has its own promoter transcribing into the tetracycline gene(s)<sup>21</sup>. We therefore propose that a previously unknown promoter which transcribes in the leftward direction exists on the tetracycline gene side of the *Hind*III site of pBR322. It is necessary to postulate the existence of this promoter to explain the expression of the HA gene in pBR322/FPV 604(L), pWT121/FPV 412(L) and pWT111/FPV 503(L). The possibility that there is a pseudo-promoter sequence in the HA gene itself, for example the (T<sub>19</sub>):(A<sub>19</sub>) sequence, is ruled out as the plasmid pBR322/FPV 605(R) does not express its HA gene (Table 1).

This postulated promoter would explain the transcription of the HA gene in the L-orientated plasmids. How is translation explained? Inspection of the nucleotide sequence around the tetracycline-HA gene junction of pBR322/FPV 604(L) reveals nonsense triplets in all three translation phases<sup>22</sup>. Thus the bacterial translational system is probably recognising a nucleotide sequence on the HA mRNA itself and initiating protein synthesis at the AUG of the pre-peptide. Comparison of the sequence of the untranslated region of the HA gene with that of the 3' end of prokaryotic 16S ribosomal RNA<sup>11</sup> reveals surprising complementarity (Fig. 1B). Consistent with this interpretation is the expression of HA antigen in pWT111/FPV 502(R). In this case the HA gene is in the wrong phase to be translated from the *trpE* AUG; protein synthesis initiating at the *trpE* AUG is terminated by the now in phase UGA triplet at position 23–25 in the HA gene. Thus we conclude that initiation is from the natural HA AUG at position 22–24 (Fig. 1A). Assuming equal transcription from the *trp* promoter in pWT121/FPV 411(R) and pWT111/FPV 502(R) and that the products have equal antigenic properties, then from the data of Table 1 we conclude that the eukaryotic ribosome binding site is

recognised with an efficiency of 20% compared to the prokaryotic site.

The fivefold difference in HA expression between pWT121/FPV 411(R) and pWT111/FPV 502(R) could be due to differences in the plasmid complement of the respective cells. To exclude this possibility the relative copy numbers of the above plasmids were determined from agarose gels of lysed whole cells<sup>9</sup> and found to be the same (data not shown).

Hitherto the *Hind*III and *Eco*RI sites of pBR322 have been assumed to be non-expression sites because of their respective situations in and upstream of the tetracycline promoter<sup>20,21</sup>. This has been thought to be of value in assessing the containment categorisation when considering the safety of experiments in genetic engineering<sup>23</sup>. In view of the above findings it is clear that the validity of these assumptions should be reassessed. It seems likely that a potential ribosome binding site on the inserted gene will be of some importance.

**Table 2** Control of HA expression from the tryptophan promoter/operator region

| Plasmid           | Addition   | HA* (ng per 50 µl) |
|-------------------|------------|--------------------|
| pWT121/FPV 411(R) | Tryptophan | 0.9                |
|                   | IAA        | 59.1               |
| pWT121/FPV 412(L) | Tryptophan | 9.6                |
|                   | IAA        | 4.9                |

Cultures (5 ml) of pWT121/FPV 411(R) and pWT121/FPV 412(L) were grown for 3 h at 37°C in M9 salts, glucose, casamino acids medium supplemented with carbenicillin (100 µg ml<sup>-1</sup>) and either IAA (20 µg ml<sup>-1</sup>) or tryptophan (100 µg ml<sup>-1</sup>). Cells were collected, lysed and assayed for HA content as described in Table 1.

\* Results normalised to same A<sub>600</sub>.

## Control of gene expression

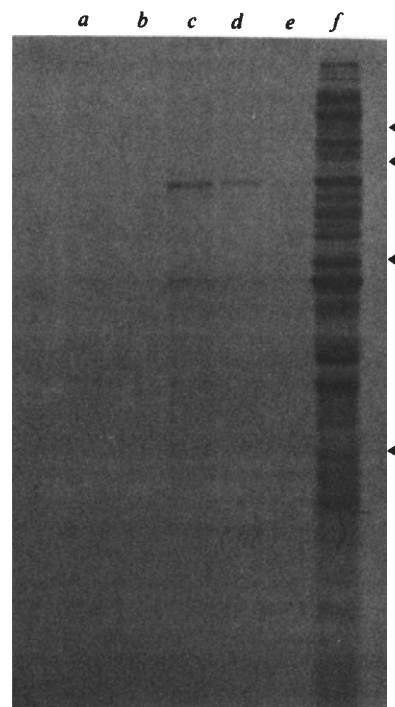
*In vivo* the tryptophan operon is controlled by the level of tryptophan<sup>24</sup> and control is best obtained in culture by using tryptophan as repressor or β-indole acrylic acid (IAA) as inducer. The latter is a competitive inhibitor that prevents tryptophan binding and thereby effectively inactivates the repressor<sup>25</sup>.

We selected a representative colony from each of the two expressing groups (in pWT121) to examine HA synthesis in the above conditions. Our initial observation was that pWT121/FPV 411(R) grew very slowly in the presence of 20 µg ml<sup>-1</sup> IAA; there was a threefold difference compared to growth in the presence of tryptophan. pWT121/FPV 412(L) grew equally well in both sets of conditions. The results of radioimmunoassay of these cultures are shown in Table 2. Because of the differences in growth rate, the values are all normalised to the A<sub>600</sub> of the culture containing pWT121/FPV 411(R) plus tryptophan. In pWT121/FPV 411(R), HA production is stimulated 65-fold when the *trp* operon is derepressed. In contrast, IAA decreased HA synthesis in pWT121/FPV 412(L). Thus increased transcription from the *trp* promoter is competing with transcription from the presumed promoter near or around the tetracycline gene and reducing expression in the leftward direction.

The effects of repression and induction on HA synthesis are also seen in Fig. 3. As little as 5 µg ml<sup>-1</sup> tryptophan was sufficient for maximal repression. On the other hand, because of its effect on growth, the induction characteristics of IAA were more complex. Maximal induction occurred at IAA concentrations of 4–8 µg ml<sup>-1</sup> and HA synthesis was stimulated 120-fold over the repressed level.

Finally, we examined the immunoprecipitable products in cells containing various plasmids after induction or repression of *trp* transcription. At 3 h after addition of either IAA or tryptophan to cultures of such cells, aliquots were pulse-labelled with <sup>35</sup>S-methionine and the immunoprecipitable proteins separated by SDS-polyacrylamide gel electrophoresis. No immunoprecipitable proteins were present in induced cells containing pWT121 or in repressed cells containing pWT121/FPV 411(R) (Fig. 4a and b, respectively). However, induced cells containing pWT121/FPV 411(R) or pWT111/FPV 502(R) and repressed cells containing

**Fig. 4** Immunoprecipitation of the haemagglutinin-like protein. Cultures of *E. coli* K12 HB101 containing the plasmids pWT121, pWT121/FPV 411(R), pWT121/FPV 412(L) and pWT111/FPV 502(R) were grown in M9 salts, glucose, casamino acids medium containing carbenicillin ( $100 \mu\text{g ml}^{-1}$ ) and either IAA ( $10 \mu\text{g ml}^{-1}$ ) or tryptophan ( $40 \mu\text{g ml}^{-1}$ ). After a 3-h period, 5-ml samples were removed and pulse-labelled for 10 min at  $37^\circ\text{C}$  with  $50 \mu\text{Ci}$  of  $^{35}\text{S}$ -methionine. The labelling was terminated by the addition of 20 ml cold M9 medium, the cells pelleted by centrifugation at  $12,000g$  for 10 min at  $4^\circ\text{C}$  and lysed, in a final volume of 1 ml, as described in Fig. 2A. Immunoprecipitations were performed as follows: to  $400 \mu\text{l}$  *E. coli* extract was added  $2 \mu\text{l}$  10% Nonidet P-40 (NP-40),  $4 \mu\text{l}$  ( $2 \mu\text{g}$ ) normal rabbit IgG and the mixture incubated for 60 min at  $20^\circ\text{C}$ . An aliquot of  $10 \mu\text{l}$  of a 10% suspension of killed *Staphylococcus aureus*<sup>31</sup> (Cowan 1 strain) was then added and incubation continued for a further 15 min. The resulting immunoprecipitate was pelleted and discarded. To the supernatant was added  $2 \mu\text{g}$  rabbit anti FPV-HA and, after 60 min at  $20^\circ\text{C}$ ,  $10 \mu\text{l}$  of *S. aureus*. This immunoprecipitate was pelleted 15 min later and washed twice with a solution containing 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4 and 0.05% NP-40. Radioactive proteins were eluted from the complex with  $30 \mu\text{l}$  SDS buffer, heated  $90^\circ\text{C}$  for 2 min and electrophoresed on a 12.5% polyacrylamide-SDS gel<sup>32</sup> which was then dried and autoradiographed. The figure shows the immunoprecipitable proteins from cells containing the following plasmids: a, pWT121 + IAA; b, pWT121/FPV 411(R) + *trp*; c, pWT121/FPV 411(R) + IAA; d, pWT111/FPV 502(R) + IAA; e, pWT121/FPV 412(L) + *trp*; f contains an extract from induced cells containing pWT121/FPV 411(R). The positions of conalbumin (MW 76,000), bovine serum albumin (69,000), ovalbumin (43,000) and carbonic anhydrase (30,000) are indicated by the arrows.



pWT121/FPV 412(L) all produced a band of MW 61,000 (Fig. 4c, d and e respectively). Our experimental design predicted that the HA-like protein synthesised from pWT121/FPV 411(R) would have a MW of 69,168 and that the products from pWT121/FPV 502(R) and pWT121/FPV 412(R) would be 26 amino acids smaller at the N-terminus. As the three products are in fact the same size, it seems probable that the primary gene product has been processed and a portion of the polypeptide removed. The same results were obtained when the products of these plasmids were examined in minicells (unpublished results). Clearly the mechanism of the processing and the possible involvement of the pre-HA sequence need further investigation.

## Conclusions

We have demonstrated the feasibility of producing controlled amounts of influenza antigenic determinants by genetic engineering. Obviously further analysis is necessary to characterise the protein product and a number of questions remain unanswered. First, for example, initiation of protein synthesis from the prokaryotic-like ribosome binding site on the HA will result in a polypeptide having a eukaryotic leader sequence. This sequence is thought to function in the transmembrane movement of the HA *in vivo*<sup>26</sup>; we may question whether this sequence is recognised by the regulatory systems for export in bacteria. Second, what effect do the  $(T_{19})$ : $(A_{19})$  region, resulting from the oligo(dT) primer and the poly(A) tail of the vRNA and situated between the AUG of *trpE* and the HA sequence, or the  $(Phe)_6$  oligopeptide resulting from its translation, have on transcription and translation respectively? Homopolymeric nucleotide regions such as this are one of the signals implicated in transcription termination<sup>27</sup>; thus the  $(T_{19})$ : $(A_{19})$  region may have a polar effect on HA production. Similarly, the  $(Phe)_6$  region may result in depletion of charged tRNA<sub>Phe</sub> and slow ribosome movement along the mRNA. Clearly examination of HA production by plasmids where this region has been removed is essential.

The absolute quantity of HA-like protein produced may also be estimated. The estimates based on immunological detection cannot be precise and are probably minimum values since if there are differences from the natural antigen it seems likely that the bacterial product would have fewer antigenic determinants or lower affinity for the antiserum. Based on our maximum yield of 60 ng per  $50 \mu\text{l}$  bacterial lysate (Table 2) and a protein concentration of  $160 \mu\text{g ml}^{-1}$  for the same lysate we arrive at a figure of 0.75% of total protein. By comparison, the quantities of induced proteins can be determined by densitometry from autoradiographs of gels containing proteins from induced and repressed cells. This shows that the 61,000 MW protein is 2–3% of total protein synthesis (unpublished results). Neither of these values was as high as expected. Hallowell and Emtage<sup>28</sup> have recently cloned an *E. coli* HindIII fragment containing the *trp* promoter and the complete *trpE* gene in pBR322; in induced conditions this plasmid was capable of specifying up to 30% of total protein as anthranilate synthetase<sup>28</sup>. Our values however are similar to those reported for the synthesis of ovalbumin-like proteins under the control of the *lac* promoter<sup>29,30</sup>; that is, the level of expression of the eukaryotic genes is only about 10% of the expected. Whether this is due to limiting amounts of some tRNA species as proposed by Fraser and Bruce<sup>30</sup> or to the type of transcription termination discussed above remains to be seen.

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## LETTERS

# A search for neutral hydrogen absorption in the double quasar 0957+561

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**We report here the results of a search for neutral hydrogen absorption in the spectrum of 0957+561. This source consists of two quasars with similar optical spectra and Mg II absorption redshifts, and an extended region containing most of the radio flux. We place limits on the optical depth of intervening hydrogen of 0.02 and 0.23, depending on whether such hydrogen covers all of the source or only the compact components.**

The quasars 0957+561 A, B were discovered by Porcas *et al.*<sup>1</sup> as two blue objects of nearly equal magnitude, separated by 6 arc s. Walsh *et al.*<sup>2</sup> have suggested that these two objects, having the same redshifts and essentially identical optical spectra, may be one source split into two images by a gravitational lens. High resolution maps at 6 cm by Roberts *et al.*<sup>3</sup> and Pooley *et al.*<sup>4</sup> do not disprove this model, but an equally likely and possibly simpler interpretation is that the source is a true double quasar, with one component presently ejecting relativistic plasma<sup>3</sup>. Spectroscopic observations by Weymann *et al.*<sup>5</sup> on the multiple mirror telescope give heliocentric vacuum Mg II absorption redshifts of 1.3911 for A and 1.3912 for B.

The radio absorption line observations reported here were conducted on the 300-ft telescope of the National Radio Astronomy Observatory in Green Bank, West Virginia, during a two week period in June 1979. The 515–740 MHz dual channel receiver, with a system temperature of about 130 K, was used to search the redshift range 1.388–1.394. Spectra were processed by the Mark III autocorrelator running in a parallel mode, with 192 channels devoted to each of the two independent receivers. A bandwidth of 2.5 MHz was used, yielding a channel spacing of  $\sim 3.5 \text{ km s}^{-1}$ . The system was operated in the total power mode with off-source scans taken before and after on-source scans. The total on-source integration time was  $\sim 2\text{h}$  for each receiver, and the two receivers were averaged together in the data reduction. The resulting spectrum is shown in Fig. 1, and shows no evidence for neutral hydrogen absorption lines deeper than 0.02 K. The spectrometer system was checked by observing the H II region W43. The 222 $\alpha$  hydrogen recombination line at 595.01 MHz was seen at the expected intensity.

Continuum antenna temperature measurements were made at 594 MHz with the same receiver; however, there is a confusing source, 4C+55.19, about 16 arc min south-east of the quasars (2/3 power point in the beam), of unknown 594 MHz

flux. This source is clearly seen at the extreme edge of a full field VLA map of 0957+561 at 20 cm (Roberts, personal communication), but it is so far off-centre that the flux is unreliable. We estimate that the 594 MHz flux of 0957+561 is about 1 Jy, based on fluxes at 20 cm (Roberts, personal communication) and at 73 cm (Pooley *et al.*<sup>4</sup>). The corresponding 300 ft antenna temperature is  $\sim 0.9 \text{ K}$ . The flux at 6 cm was measured with the 6–25 receiver, which has a system temperature of about 80 K, and found to be  $230 \pm 20 \text{ mJy}$ . Both measurements were made in July 1979.

The 6 cm VLA map of Roberts *et al.*<sup>3</sup> has an angular resolution of 0.8 arc s, and shows a pair of unresolved sources coincident with the optical quasars A and B (A being northernmost). In addition, there is a double, extended source 2–5 arc s north-east of A, and a probable extended source 5 arc s south-west of A, these sources being designated C, D, and E respectively by Roberts *et al.*<sup>3</sup> If the entire complex is covered by the absorber, the 0.02 K absorption limit and the 0.9 K estimated antenna temperature place an upper limit on the optical depth of  $\tau \leq 0.02$ . As we do not know where the optical lines are formed, however, this assumption may not be valid. A more conservative assumption is that only the compact sources A and B, which show almost identical Mg I, Mg II, and Fe II optical absorption lines, are covered. Of the total VLA flux of 220 mJy, source A contributed 36 mJy, and source B 30 mJy. Pooley *et al.*<sup>4</sup> report a 408 MHz flux of 120 mJy for A, and an upper limit of 50 mJy for B. Interpolating the fluxes between these two frequencies, we estimate that the combined 594 MHz flux of A and B lies between 100 and 150 mJy. In this case, our limit on the optical depth would be  $\tau \leq 0.23$ .

Both optical and radio absorption lines have been detected in the spectra of other compact sources. The Mg II and Fe II optical lines in the BL Lac object AO0235+164 and the quasar 1331+170 are the most similar to those in 0957+561; optical and radio results for these three are summarised in Table 1.

The equivalent widths of the optical lines in 0957+561 are intermediate between those of AO0235+164 and 1331+170. If the absorbing material covers only A and B, the resulting optical depth limit of  $\tau \leq 0.23$  is consistent with the known optical depths of the H I absorption lines in the other two sources. Alternatively, if the absorber covers some or all of C, D, and E, the peak optical depth of 0.02 may imply a higher spin temperature or a lower density for the hydrogen in this absorber than for that in the other two absorption systems.

To place a limit on  $n_{\text{H}}$ , the column density of hydrogen, we assume a rest frame line width of  $10 \text{ km s}^{-1}$ . With spin temperature  $T_s$ , the limits are  $(n_{\text{H}}/T_s) < 4 \times 10^{18} \text{ H cm}^{-2} \text{ K}^{-1}$  for  $\tau < 0.23$ , and  $n_{\text{H}}/T_s < 4 \times 10^{17} \text{ H cm}^{-2} \text{ K}^{-1}$  for  $\tau < 0.02$ .

The optical lines are unresolved and highly saturated, so that only approximate information about column densities of Mg II can be extracted. From the equivalent widths of Weymann *et al.*<sup>6</sup> the doublet ratio of the Mg II lines is 0.92 for source A and 1.04

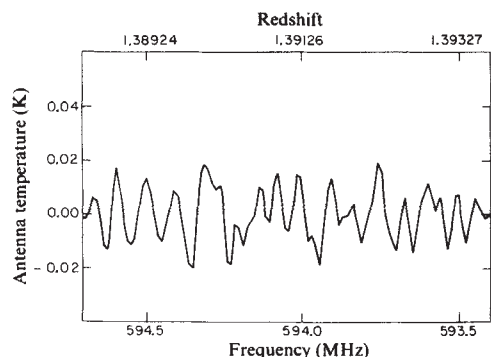
**Table 1** Comparison of 0957+561 with similar absorption systems

| Source     | $z_{\text{abs}}$ | $W_{\lambda}$ (rest frame) |                |                | Fe II<br>2,599 | H I width<br>(observed frame)<br>( $\text{km s}^{-1}$ ) | Optical depth<br>at 21 cm             | Refs |
|------------|------------------|----------------------------|----------------|----------------|----------------|---------------------------------------------------------|---------------------------------------|------|
|            |                  | Mg II<br>2,796             | Mg II<br>2,803 | Fe II<br>2,586 |                |                                                         |                                       |      |
| 0957+561   | A                | 1.391                      | 2.0            | 2.2            | 1.0            | —                                                       | <0.02*, <0.23†                        | 1–5  |
|            | B                | 1.391                      | 2.1            | 2.0            | 0.75           |                                                         |                                       |      |
| AO0235+164 | 0.524            | 2.42                       | 2.34           | 1.46           | 1.79           | Total $\approx 50$<br>Structure $\geq 6$                | 0.71 deepest line<br>0.53 2nd deepest | 6–8  |
| 1331+170   | 1.776            | 1.30                       | 2.16‡          | 0.65           | 0.54           | 22.2                                                    | 0.02*                                 | 9–11 |
|            | 1.785            | 2.15‡                      | 1.04           | —              | —              |                                                         |                                       |      |

\* Absorber covering all of source.

† Absorber covering A and B components only.

‡ Lines blended.



**Fig. 1** Antenna temperature as a function of heliocentric redshift and frequency. The spectrum represents about two hours of on-source integration time, and has no absorption features greater than 0.02 K.

for B. Since the doublet ratio decreases from 2 to 1 as the ion density increases, we interpret the A value of 0.92 as a reflection of measurement uncertainties, and assign error limits to the B ratio of  $1.04^{+0.08}_{-0.04}$ . The resulting upper limit of 1.12 in the doublet ratio of Mg II corresponds to a lower limit on the column density of  $n_{\text{MgII}} > 10^{15} \text{ cm}^{-2}$ . For  $\tau < 0.23$  and  $T_s > 10 \text{ K}$  this limit is consistent with cosmic abundances; for  $\tau < 0.02$  spin temperature would have to be  $> 100 \text{ K}$ . These limits are, of course, strongly dependent on the equivalent widths of the unresolved optical absorption lines.

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## Emission feature $\lambda 3,470$ of VV Puppis may be a cyclotron line

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**The possibility that the unidentified emission feature  $\lambda 3,470$  in the spectrum of VV Puppis is a cyclotron line is discussed and a theory to explain such an origin is presented.**

VV Puppis displays a large linear and circular polarisation  $\bar{P}_{\text{max}} = q_{\text{max}} \approx 16\%$  (ref. 1) showing it to be an object of the AM Herculis type. These objects (AM Her, AN Ursae Majoris, VV Pup and 2A0311–227) are close binary systems containing an outflowing non-degenerate star and an accreting degenerate dwarf. Stars of the AM Her group have no eruptions and probably belong to the nova-like class. The polarisation of VV Pup light changes regularly with an orbital period of  $\sim 100 \text{ min}$ . During  $\sim 60\%$  of this period VV Pup usually

increases its luminosity. Its magnitude varies from  $m_{\text{min}} = 14.4$ –18 to  $m_{\text{min}} - \Delta m$  mag where  $\Delta m = 0$ –2.5. There seems to be no clear correlation between the  $m_{\text{min}}$  and  $\Delta m$  variations. The maximum positive circular polarisation  $q_{\text{max}}$  is observed in the phases of the orbital period when VV Pup is photometrically bright. In the phases of minimum light the circular polarisation is weaker and negative. The magnitude of  $q_{\text{max}}$  appears to change from one observation to another.

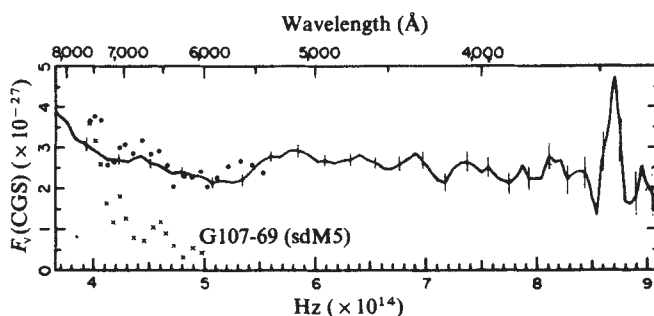
Although the VV Pup spectrum usually has strong H and He II emission lines, Angel *et al.*<sup>2</sup> have observed that this star sometimes has an unusual state of steady low luminosity with no strong H and He II emission. According to a more detailed description of these observations<sup>3</sup>, in March and April 1977 VV Pup was in two different states. In March the magnitude in the minimum phases was small  $m_{\text{min}} \approx 17.7$ , brightening was large  $\Delta m \approx 2.5$  and the H and He II emission lines were strong. The average H $\beta$  flux was about 1% of the continuum 3,500–6,000 Å flux. In April the minimum light was approximately the same, but brightening was small  $\Delta m \leq 0.5$  and the spectra has no strong H, He or some other elements emission (H $\beta$  flux did not exceed 0.02% of the 3,500–6,000 Å continuum). Nevertheless, a strong unidentified emission line near 3,470 Å was statistically significant<sup>3</sup> in both spectra obtained by a single-channel prism scanner of the Steward 2.3-m telescope (Fig. 1) and a Kitt Peak 4-m intensified image-dissector scanner.

The unidentified line cannot be an atomic line of some element, because other lines of such an element would also be observed. The feature has been observed using different equipment, so the coincidence of any systematic errors or 'glitches' seems unlikely. The feature corresponded to energy flux of about 3% of the 3,500–6,000 Å continuum, that is, it was very strong. When VV Pup was in the active state (March 1977), the emission feature  $\lambda 3,470$  was much smaller<sup>3</sup>.

One possibility is that the strong emission feature  $\lambda 3,470$  is a cyclotron line. The magnetic field  $B$  corresponding to the wavelength of this line is equal to  $3.1 \times 10^8 \text{ G}$ . The accuracy of this determination depends on the spectral width of the feature, the estimated error is  $\Delta B = (\Delta \lambda / \lambda) \times B \approx 10^7 \text{ G}$ .

This magnetic field is likely to belong to the degenerate component of VV Pup. The presence of a magnetised degenerate dwarf in the AM Her system was predicted when the origin of the large circular and linear polarisation of AM Her light was discussed<sup>4</sup>. Two different sources of polarised light were proposed: (1) the optically thick cyclotron or synchrotron emitting accretion column<sup>4–6</sup> and (2) the photosphere of a magnetised degenerate dwarf<sup>7</sup>. In both cases the dwarf's magnetic field was estimated as  $\geq 10^8 \text{ G}$ . There is a good agreement between this estimation and the value  $3.1 \times 10^8 \text{ G}$  following from our interpretation. A study of polarisation in the spectral region near  $\lambda 3,470$  would be required for a positive confirmation. Nevertheless, a short theoretical verification of this identification seems to be necessary.

The cyclotron emission, which is responsible for a cyclotron line, cools the emitting plasma very effectively. The necessary heating may arise from the accretion process when the gravitational energy of the accreted plasma is released near the surface of a compact star. The cyclotron emitting plasma accreted by a



**Fig. 1** The spectrum of VV Puppis obtained in April 1977 by Liebert *et al.*<sup>3</sup>.



magnetised neutron star was considered<sup>8</sup> for the conditions of an X-ray pulsar. The emission features corresponding to cyclotron harmonics were found to be present in the X-ray spectrum. Later the radiation of the accreting degenerate dwarf was considered in more detail<sup>9-12</sup>, when the optical depth of the emitting region is sufficiently large with respect to the cyclotron absorption and the optical cyclotron continuum is radiated.

In April 1977, the VV Pup continuum source in 3,500–6,000 Å band was accepted to be the degenerate dwarf's surface with temperature 9,000 K (ref. 3). Therefore, at this low state the shock region was likely to be optically thin for cyclotron radiation and only narrow cyclotron line was radiated. The low luminosity states seem to correspond with the regime of weak accretion. The variations of the accretion result, for example, from the changes of the position of the outflowing star inside the dwarf's magnetosphere. The distance from VV Pup is about 80–150 pc. Using the composite energy distribution<sup>3</sup> one can estimate luminosities  $L_{\text{cont}} = (0.5-2)10^{30} \text{ erg s}^{-1}$  and  $L_{\text{cyc}} = (2-6)10^{28} \text{ erg s}^{-1}$  in the low state. It seems natural to assume that nearly all the gravitational energy released was radiated in the cyclotron line. To provide for the observed  $L_{\text{cyc}}$ , a weak accretion at a rate of  $\dot{M} \approx 3 \times 10^{11} \text{ g s}^{-1}$  ( $M_{\text{dwarf}}/M_{\odot} \times (5 \times 10^8 \text{ cm}/R_{\text{dwarf}})^2$ ) is necessary (it is the low limit for accretion rate at low state). The radius of the Alfvén surface seems to be much larger than the distance between the VV Pup's components. This means that the dwarf's magnetic field at every point determines the dynamics of the transferring plasma. Assuming the dipole field configuration, one can estimate the area  $S$  of the dwarf's surface where the accretion occurs. If the radius of the outflowing region on the non-degenerate component is of the order of its radius  $\sim 2 \times 10^{10} \text{ cm}$  and the distance between the dwarf and the outflowing region is about  $2.5 \times 10^{10} \text{ cm}$ , then  $S \approx 5 \times 10^{15} \text{ cm}^2$ . The supposed cyclotron line should arise in the plasma heated by the standoff shock above this area. What are the conditions necessary for the line origin?

(i) The region of heated plasma should not be optically thick with respect to the cyclotron absorption. The corresponding optical depth in the direction perpendicular to the stellar surface is equal to<sup>13</sup>

$$\tau_{\text{cyc}}(\nu) \approx (2\pi)^{1/2} \langle nd \rangle \cdot \frac{e^2}{m_e v_e \nu_B} \cdot \exp \left[ - \left( \frac{c \Delta \nu}{\sqrt{2} v_e \nu_B} \right)^2 \right] \quad (1)$$

where  $v_e$  is the thermal velocity of the electrons,  $e$  and  $m_e$  are their charge and mass,  $\nu_B$  is the cyclotron frequency and  $\Delta \nu$  is  $\nu - \nu_B$ . The parameter  $\langle nd \rangle$  is the mean product of the electron concentration  $n$  and the height  $d$  of the heated region. The value of  $v_e$  may be estimated from the condition that the observed mean linewidth is larger than Doppler broadening  $\Delta \lambda / \lambda_B \approx 3 \times 10^{-2} \geq 2v_e/c$ . Hence  $v_e \leq 5 \times 10^8 \text{ cm s}^{-1}$  and the electron temperature  $T_e \leq 10^6 \text{ K}$ . According to equation (1), the heated region is optically thin if  $\langle nd \rangle \leq 10^{15} \text{ cm}^{-2}$ . It is easy to check that for the cyclotron line all other absorption processes are not as essential as this one. For the equilibrium to set in between the pressure of the infalling plasma  $\sim Mv_n/S$  and the thermal pressure inside the hot region  $\sim 2nkT$  it is necessary that  $n = 10^{14} \text{ cm}^{-3}$  for  $T = 10^6 \text{ K}$ . Therefore,  $d$  should be  $\sim 10 \text{ cm}$ .

(ii) The plasma heated by the stand-off shock has to provide for the observed luminosity of the cyclotron line

$$L_{\text{cyc}} = \frac{B^2}{4\pi} \sigma_T \left( \frac{v_e}{c} \right)^2 S \langle nd \rangle \\ \approx 10^{14} \left( \frac{B}{3 \times 10^8 \text{ G}} \right)^2 \cdot \left( \frac{T}{10^6} \right) \cdot \left( \frac{S}{5 \times 10^{15} \text{ cm}^2} \right) \text{ erg s}^{-1} \quad (2)$$

where  $\sigma_T$  is the Thomson cross-section of the electron. The value of  $L_{\text{cyc}}$  estimated from equation (2) agrees with the observed luminosity  $\sim 10^{29} \text{ erg s}^{-1}$  if  $\langle nd \rangle$  is just the same, that is,  $10^{15} \text{ cm}^{-2}$ .

(iii) The magnitude of the parameter  $\langle nd \rangle$  should be sufficiently large for the shock region to ensure the release of the

total gravitational energy. For the Coulomb braking of protons by plasma with concentration  $\sim 10^{14} \text{ cm}^{-3}$  it is necessary that  $\langle nd \rangle_{\text{Cul}} = 10^{19} \text{ cm}^{-2}$  ( $M_{\text{dwarf}}/M_{\odot})^2 \times (5 \times 10^8 \text{ cm}/R_{\text{dwarf}})^2$ . This magnitude is too large compared to  $10^{15} \text{ cm}^{-2}$  following from points (i) and (ii) above. However, one should take into account that the proton accretion flow is accompanied (and compensated) by the electron accretion flow. In this case, the braking appears to be much more effective due to the generation of sound and Langmuir oscillations and the interaction with the plasma turbulence<sup>13</sup>.

Thus one can conclude that the observed<sup>3</sup> emission feature  $\lambda 3,470$  may be a cyclotron line arising due to the accretion at a rate  $\sim 3 \times 10^{11} \text{ g s}^{-1}$  in the heated region with  $S \approx 5 \times 10^{15} \text{ cm}^2$ ,  $d \approx 10 \text{ cm}$ ,  $n \approx 10^{14} \text{ cm}^{-3}$ ,  $T_e \approx 10^6 \text{ K}$  above the surface of the degenerated dwarf with  $B \approx 3.1 \times 10^8 \text{ G}$ .

Four implications follow from this conclusion:

(1) Some of the energy emitted in the cyclotron line should be absorbed by the surface of the degenerated dwarf giving rise to a hot spot. To find the exact relation between  $L_{\text{spot}}$  and  $L_{\text{cyc}}$  and the continuum spectrum of the hot spot one should solve the problem of radiation transport in the presence of a strong magnetic field and accretion heating. Nevertheless, it seems clear that all the released accretion energy is radiated in the cyclotron line and UV continuum when VV Pup is in the low accretion state (low steady luminosity).

(2) The increase of the accretion rate leads to increasing temperature and concentration in the heated region. It becomes optically thick for the cyclotron line and, as a result, the cyclotron continuum is emitted. The periodic brightening of VV Pup in the active state is thought to be associated with this continuum. Similarly, the increased accretion results in the strong H and He II emission. Thus one may predict an anticorrelation between the periodic brightening and the strong H and He II emission on the one hand, and the presence of the cyclotron line, on the other.

(3) It seems clear from the above discussion that the variation of the cyclotron line with the orbital period 100 min may be expected. It should be brighter in phases corresponding to the brightening in the active state. If there are two active magnetic poles<sup>14</sup> with slightly different magnetic fields, the frequency of the cyclotron line should vary with the phase.

(4) It may be possible to observe the cyclotron line in the AM Her and AN Ursae Majoris spectra when they have low luminosity. The cyclotron frequency is equal to  $\nu_B = 2.8 \times 10^{14} (B/10^8 \text{ G}) \text{ Hz}$  ( $\lambda_B = 10,700 \times (10^8 \text{ G}/B) \text{ Å}$ ). It would also be of interest to search for a cyclotron line in the spectrum of the nova-like MV Lyra. For this star, a small variable linear and circular polarisation of 2% have been recorded<sup>15</sup>. In the spectral observations, the disappearance of the strong H and He II emission was discovered. It may be similar to the disappearance of strong H and He II emission from VV Pup. Thus MV Lyra can be considered as a candidate for the AM Her group.

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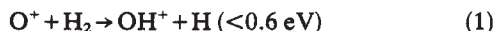
# Hot hydrogen in the exosphere of Venus

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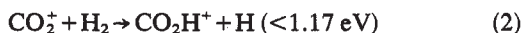
The Ly  $\alpha$  measurements of the hydrogen corona of Venus by Mariners 5 (ref. 1) and 10 (ref. 2) have been shown to be consistent with a two temperature component model. Bertaux *et al.*<sup>3</sup> have successfully fitted the Venera 9 exospheric Ly  $\alpha$  data with an elevated (500 K) single temperature; however, they also indicated that their data are not inconsistent with a two component model. Various source mechanisms have been proposed to explain the 'hot' ( $\sim 1,000$  K), energetic component of the hydrogen corona. Here we use recent results from the Pioneer Venus Orbiter (PVO)<sup>4</sup> to establish the major sources of this hot hydrogen population.

The most viable of the suggested source mechanisms were reviewed and re-evaluated by Kumar *et al.*<sup>5</sup>, who concluded that the exothermic reaction,



is the major source of the 'hot non-thermal' hydrogen. The subsequent recombination of OH<sup>+</sup> produces extremely energetic ( $\sim 8$  eV) hydrogen which makes only an insignificant contribution to the observed hot hydrogen, although it is the major contributor to the non-thermal escape flux.

Kumar *et al.*<sup>5</sup> also indicate that the reaction



can also make a modest contribution to the non-thermal population. Sze and McElroy<sup>6</sup> considered the charge exchange reaction:



and Kumar *et al.*<sup>5</sup>, Chamberlain<sup>7</sup> and Stewart<sup>8</sup> considered:



where the hot hydrogen ion could be a solar wind ion or a hot ionospheric ion. These two charge exchange reactions were found to be unimportant for the hot hydrogen population, although reaction (4) was found to contribute to the non-thermal escape flux.

The very comprehensive ionospheric data base, which is now becoming available from the instruments on board the PVO<sup>4</sup> provides a new opportunity for a re-evaluation of the various proposed source mechanisms for the hot hydrogen corona. This letter has two purposes: first, to point out that reactions (3) and (4), as well as (1), are, in light of the PVO results, important sources of hot H from the nightside. Previous studies have never really explained why there was almost as much hot hydrogen observed by Mariner 5 (ref. 1) on the nightside of Venus as there is on the dayside. Ferrin<sup>9</sup> indicated that horizontal transport of hot hydrogen from the dayside might contribute, but he was puzzled by the fact that the nightside non-thermal H seems to be even hotter than the dayside one (1,500 K on the nightside and 1,025 K on the dayside according to Anderson<sup>1</sup>). We will show that the relatively large H<sup>+</sup> and O<sup>+</sup> densities<sup>10</sup> and high ion temperatures<sup>11</sup> ( $\sim 5,000$  K) observed by PVO on the nightside enhance the importance of reactions (3) and (4). Second, we re-evaluate the sources of hot H on the dayside of Venus, in light of the PVO results and conclude that the sources previously proposed are adequate.

Kumar *et al.*<sup>5</sup> calculated column production rates for various potential sources of hot hydrogen to assess their relative importance. To evaluate the hot hydrogen density at the exobase one must take into account collisions of the hydrogen atoms with the cold background gas. Ferrin<sup>9</sup> accomplished this by using Monte Carlo calculations and a multistream approach. He showed, by assuming a hard sphere collision model, that the inclusion of such collisions and the subsequent energy cascading results in significant enhancements of the calculated hot hydrogen densities. In the present work, we used a two stream approach, employed widely in radiative transfer<sup>12</sup> and photoelectron calculations<sup>13</sup>, to evaluate the hot hydrogen fluxes and densities on Venus. The equations solved are:

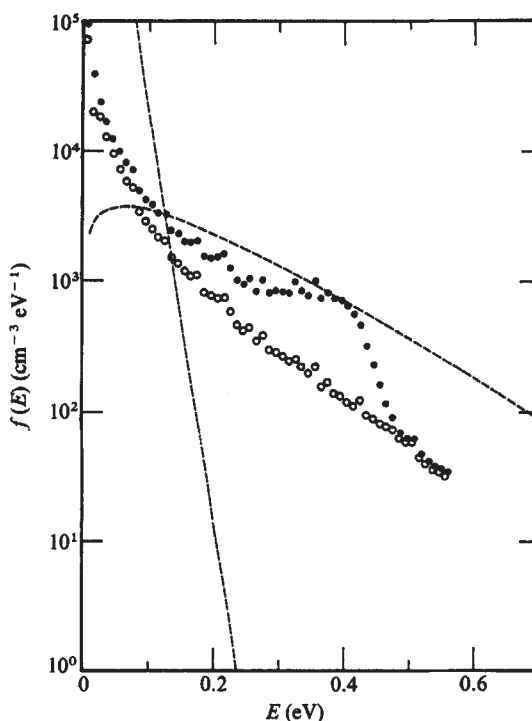
$$\begin{aligned} \langle \cos \theta \rangle \frac{d\phi^+(E, z)}{dz} &= -\sum_i n_i \sigma_i \phi^+(E, z) + \frac{P}{2} + P^+ \\ -\langle \cos \theta \rangle \frac{d\phi^-(E, z)}{dz} &= -\sum_i n_i \sigma_i \phi^-(E, z) + \frac{P}{2} + P^- \end{aligned} \quad (5)$$

where  $\phi^+(E, z)$  is the upward directed hot hydrogen flux;  $\phi^-(E, z)$  the downward directed hot hydrogen flux;  $n_i(z)$  the  $i$ -th scattering atmospheric constituent;  $z$  the altitude;  $\sigma_i$  the total scattering cross-section for species  $i$ ;  $\langle \cos \theta \rangle$  the mean of the cosine of the angle of the hydrogen velocity vector with respect to the vertical (assumed to be 0.5, corresponding to an isotropic distribution);  $P$  the hot hydrogen production rate due to the various sources under consideration (assumed isotropic); and  $P^*$  the 'production' rates at a given energy due to cascading from collisions at higher energies.

The energy distribution function,  $f(E, z)$ , can be determined from the calculated flux values, through the following relationship:

$$f(E, z) = \{\phi^+(E, z) + \phi^-(E, z)\} / v(E) \quad (6)$$

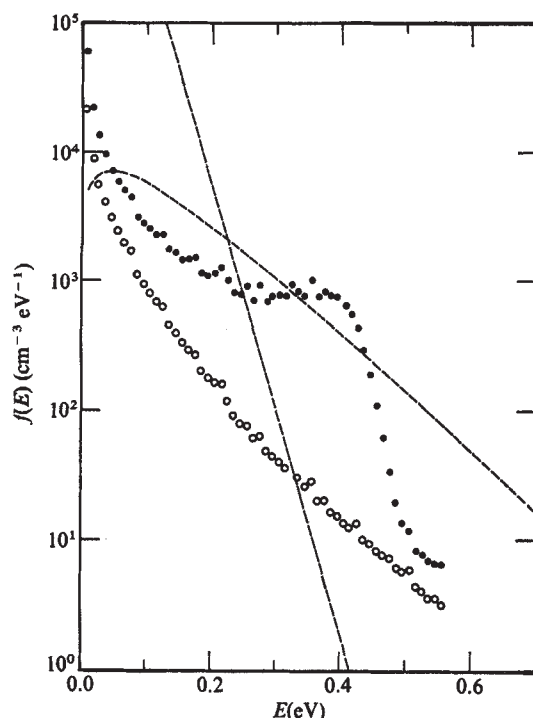
where  $v(E)$  is the hydrogen velocity corresponding to energy  $E$ .



**Fig. 1** The total calculated nightside density distribution function (●) is shown. ○, The distribution function including only the charge exchange reactions (3) and (4). The dashed lines represent the two component distribution functions deduced by Anderson<sup>1</sup> ( $n = 2 \times 10^5 \text{ cm}^{-3}$  and  $T = 150 \text{ K}$  for the cold and  $n = 1 \times 10^3 \text{ cm}^{-3}$  and  $T = 1,500 \text{ K}$  for the hot component).

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**Fig. 2** The total calculated dayside density distribution function ( $\bullet$ ) is shown.  $\circ$ , The distribution function including only the charge exchange reactions (3) and (4). The dashed lines represent the two component distribution functions deduced by Anderson<sup>1</sup> ( $n = 2 \times 10^5 \text{ cm}^{-3}$  and  $T = 275 \text{ K}$  for the cold and  $n = 1.3 \times 10^5 \text{ cm}^{-3}$  and  $T = 1,025 \text{ K}$  for the hot component).

The hot hydrogen atoms were assumed to be 'lost' either by escape at the topside or through a series of energy losses due to elastic hard sphere collisions<sup>14</sup> with the background gases ( $\text{CO}_2$ , O, H), eventually becoming part of the cold hydrogen population. We have neglected the small but finite probability of energy gain through these collisions. The average energy losses in backward and forward elastic scattering events are<sup>14</sup>

$$\overline{\Delta E_b} = \frac{3mM}{(m+M)^2} E_{in} \quad \text{and} \quad \overline{\Delta E_f} = \frac{mM}{(m+M)^2} E_{in}$$

respectively and where  $m$  is the mass of an H atom,  $M$  is the mass of the target and  $E_{in}$  is the initial H atom energy.

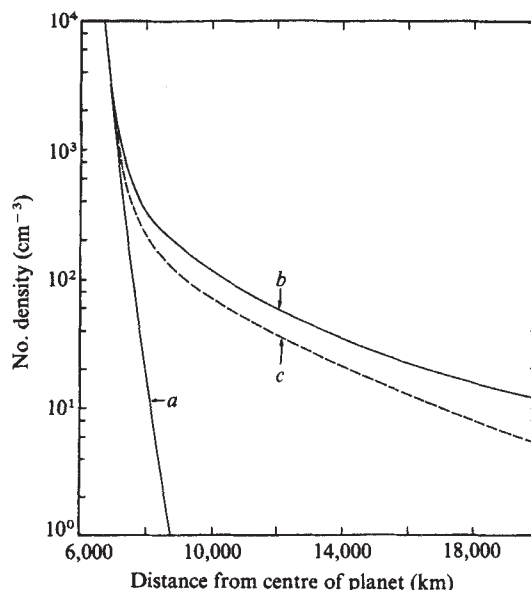
The appropriate neutral gas and ion densities as well as the ion temperatures were taken from Pioneer Venus data<sup>10,11,15</sup>, except for the neutral atomic hydrogen densities, for which the Mariner 5 estimate of Anderson<sup>1</sup> was used, and molecular hydrogen, for which the estimate of Kumar and Hunten<sup>16</sup> was used. The adopted values of the dayside Pioneer Venus data correspond to zenith angles of about  $70^\circ$ , while the nightside data correspond to zenith angles of about  $140^\circ$ . The nightside ionospheric data were taken from the outbound segment of Orbit 59. The nightside ionosphere has been found to be extremely variable from orbit to orbit; therefore, the results of these calculations should only be considered as 'representative'.

The energy grid used in the two stream calculations was 0.01 eV and the altitude grid size was variable ranging from 0.2 km below 130 km up to 5 km above 250 km. The upper boundary was at 600 km. To approximate the escaping and ballistic components of the flux, the downward directed flux,  $\phi^-$ , at the upper boundary, was assumed to be equal to the integral of the downward production above this altitude for energies greater than the escape energy (0.56 eV) and equal to the upward flux for lower energies. Although the upper boundary was set at 600 km, scattering by the background gas is not very

important above the exobase level which is at about 170 km on the dayside and at about 145 km on the nightside. Consequently, above the exobase (or critical) level the distribution functions calculated using equations (5) and (6) remain almost constant with increasing altitude, because equation (5) only takes into account 'collisional' effects. What we obtain using equation (5) are distribution functions at the exobase level. We assumed that the H atoms from reactions (1) and (2) are produced with less energy than the exothermicities of the reactions would indicate, to take into account some degree of vibrational and/or rotational excitation of the product ions. H atoms from reactions (1) and (2) were assumed to be created with a gaussian distribution; the central energies were taken to be 0.4 eV and 0.8 eV respectively, with an assumed halfwidth of 0.03 eV. The hot H atom densities calculated including the effects of scattering (and cascading) by the background gas are about a factor of three larger than those calculated assuming no scattering and cascading. This is in line with the enhancement values found by Ferrin<sup>9</sup>.

Anderson<sup>1</sup> found that he could fit the hot component of Mariner 5 nightside Ly  $\alpha$  data by assuming a Maxwellian distribution with an exobase density and temperature of  $1 \times 10^3 \text{ cm}^{-3}$  and 1,500 K respectively. The energy distribution of the total hot hydrogen densities calculated for night-time conditions are shown in Fig. 1; for comparison the contribution due only to reactions (3) and (4) is shown with open circles, and the 'hot' and 'cold' Maxwellian components deduced by Anderson<sup>1</sup> are indicated by dashed lines. These calculations indicate that the charge exchange reactions (3) and (4) are the source of about 50% and reaction (1) is the source of the other 50% of the night-time 'hot' hydrogen densities. Overall the calculated distribution function agrees reasonably well both in magnitude and shape with the deduced values in the energy range of 0.12 eV (the crossover point with the cold component) and 0.56 eV (the escape energy). The fact that the calculated distribution is not exactly Maxwellian in shape is not of special concern, because the actually observed limb intensities do not uniquely require such a distribution.

The calculated dayside exobase distribution function is shown in Fig. 2 along with Anderson's dayside hot and cold Maxwellian distributions. The overall hot density is about the same as the



**Fig. 3** H number densities for the nightside are shown as functions of the distance from the centre of the planet. *a*, Corresponds to the cold Maxwellian of Fig. 1; *b*, is for both the cold and hot Maxwellians of Fig. 1; and *c*, is for the cold Maxwellian plus the non-Maxwellian of Fig. 1.

maxwellian for energies greater than about 0.22 eV and less than 0.57 eV. The charge exchange sources contribute very little to the total hot density on the dayside. The  $O^+ + H_2$  reaction is the dominant source of the nonthermal H.

Given a distribution function at the exobase level one can find the corresponding exospheric density distribution of H as a function of altitude by using Liouville's theorem<sup>17</sup>. We calculated the exospheric density distributions, using the distribution functions shown in Figs 1 and 2 and assuming (1) that the distribution functions are isotropic and (2) that there is spherical symmetry. Only ballistic and outgoing (escaping) hyperbolic orbits were included in our calculations. This should be a fairly good assumption because, for Venus, satellite particles will be rapidly removed by charge exchange with energetic solar wind or ionosheath protons. The calculated exospheric densities for the nightside are shown in Fig. 3. The total hydrogen altitude distribution (cold and hot maxwellians), has almost the same shape as density distribution *c* (the cold maxwellian plus our calculated nightside distribution), although the absolute magnitude is somewhat larger. We also calculated the total exospheric altitude distributions for the dayside but do not show them here because of space limitations and because the agreement between our calculated altitude distribution and the one deduced by Anderson<sup>1</sup> agree even better (~20%) than shown for the nightside case.

Note that, in comparing the results of these calculations with the Mariner 5 data, the atmospheric and ionospheric conditions were different during the Mariner 5 encounter and the Pioneer Venus observations and furthermore, the ionospheric densities are highly variable and only model values of  $H_2$  are available. The calculations presented here indicate, within the limitations just mentioned, that reactions (1), (3) and (4) are the major sources of the 'hot' component of Venus hydrogen corona. On the nightside the charge exchange reactions are important because the ion temperatures are high and the  $H^-$  densities are greater than on the dayside. There are preliminary indications, from the Pioneer Venus ion mass spectrometer results<sup>10</sup>, that there is a significant night-time hydrogen bulge; if the night-time 'cold' hydrogen densities are greater than the values assumed in these calculations, the importance of reaction (4) will increase further. Contrary to earlier suggestions<sup>9</sup>, most or at least a significant fraction of the nightside hot hydrogen population is apparently produced locally.

As the Pioneer Venus Ly  $\alpha$  intensity results become available, more quantitative comparisons can be carried out. The nightside Venus ionosphere is highly variable, with apparent time scales of the same order as the hot hydrogen lifetime ( $\sim 10^3$  s) and therefore, orbit to orbit comparisons between the measured Ly  $\alpha$  intensities and ionospheric parameters can be used to check the validity of the basic mechanisms suggested in this work.

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## A weak interaction model for Io and the jovian magnetosphere

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Four features of the interaction between the satellite Io and the surrounding jovian magnetosphere place constraints on the atmosphere of the satellite and on the nature of the interaction: (1) the atmosphere must be at least partly an exosphere so that sodium and other non-volatile atoms sputtered from the surface of the satellite could escape<sup>1,2</sup>; (2) the atmosphere should be dense enough for a detectable ionosphere to form showing a leading-trailing asymmetry<sup>2,3</sup>; (3) the atmosphere should provide an electrically conductive path to produce the electric currents which eventually cause the Io-related decametric radio emissions<sup>4,5</sup>; (4) the conductivity of the ionosphere should not be large enough to short out the electric field seen by the satellite, as pronounced absorption of energetic particles is observed<sup>6-8</sup>. Before the Pioneer 10 encounter it was pointed out<sup>9</sup> that erosion by the co-rotating magnetosphere caused atmospheres on the galilean satellites with surface pressures in the range  $10^{-5}$ – $10^{-9}$  mbar, if they exist, to be stable only if they are continuously replenished and therefore likely to be associated with "venting or the presence of non-hydrous ices on the surface". Alternatively, if the surface is covered with non-volatiles and/or water ice, the atmospheric pressure would be very low and controlled by sputtering due to the impact of magnetospheric plasma and energetic particles on the surface<sup>9</sup>. In any case, the interaction with the magnetosphere is likely to be 'weak' as it is for Mercury<sup>10</sup>, that is, the magnetic field and plasma sweep into and past the satellite being affected mainly by the additional inertia of newly formed ions (provided the satellite is non-conducting and non-magnetic). We substantiate these arguments here using the recent Voyager observations, and suggest that the interaction between Io and the magnetosphere can be understood if the atmosphere of Io were largely exospheric in nature and controlled by the vapour pressure of  $SO_2$  ice which covers the surface.

The discovery of volcanic activity on Io by Voyager 1 (ref. 11) confirms that venting of gas from beneath the surface must be an important source of gas in the atmosphere. By analogy with the gaseous products of terrestrial volcanoes (ignoring the  $H_2O$  and  $N_2$  which probably have an external origin) one might expect  $CO_2$  and  $SO_2$  to predominate together with smaller amounts of  $SO_3$ ,  $H_2S$ , CO, A, He,  $H_2$  and  $Cl_2$  (ref. 12). The behaviour of such volatiles, if ejected from the volcanoes on Io, can be studied in terms of a vapour pressure versus temperature diagram as shown in Fig. 1. Evidently  $CO_2$  and several other possible constituents such as  $CH_4$ , CO and  $N_2$  have such high vapour pressures that they would tend to completely dominate the atmosphere if they were present in the volcanic ejecta in any significant amounts. However, Voyager 1 observations of energetic heavy ions in the jovian magnetosphere indicate that S and O ions are dominant and C and N ions (other than those of obviously solar origin) are essentially absent<sup>6</sup>. Furthermore,  $SO_2^+$  ions and their dissociation products were detected by the plasma experiment<sup>13</sup> and an  $SO_2$  absorption feature was identified on the surface of the satellite by the IR and radiometry experiments<sup>14</sup>. Accordingly, we conclude that the gaseous emission from the Io volcanoes is almost entirely  $SO_2$  and that the other components commonly found in terrestrial volcanoes are absent or very depleted.

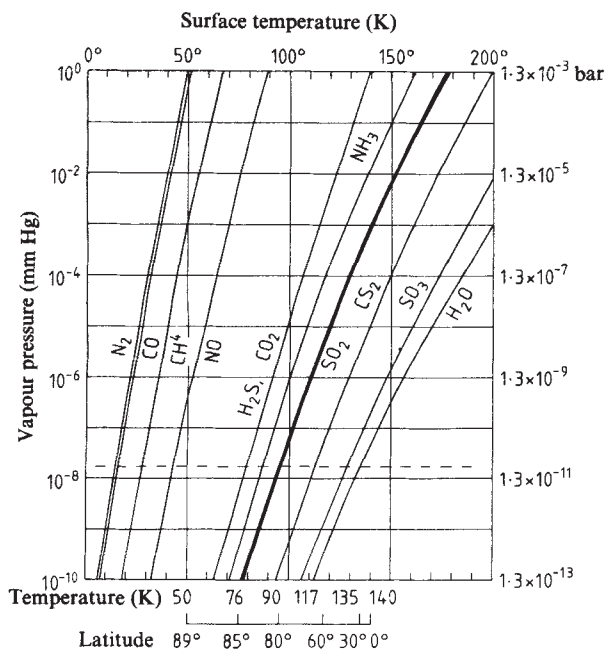
To understand how this would affect the atmosphere of Io we should consider the situation where the surface of the satellite is uniformly covered with  $SO_2$  frost and the atmospheric pressure is determined locally by the surface temperature (the effects of

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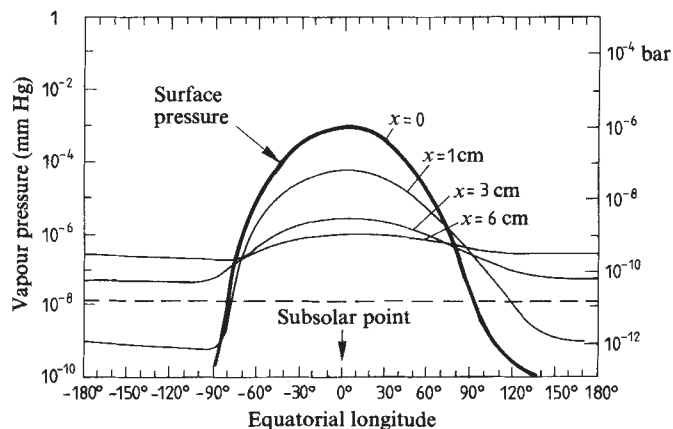


pressure gradients in inducing lateral flows are neglected). Figure 2 shows that the pressure should vary from  $\sim 1.3 \times 10^{-3}$  mbar on the equator at the subsolar point ( $T = 140$  K) to an exospheric level ( $< 2 \times 10^{-8}$  mbar) at elongations exceeding  $80^\circ$  from the subsolar point ( $T < 100$  K). This maximum pressure exceeds the upper limits obtained from occultation measurements<sup>15</sup> but as these only apply to the limb, a detectable atmosphere would not be expected. The major constituents of the atmosphere should be essentially frozen out on the dark side of the satellite and over the whole surface during eclipses. However,  $^{40}\text{Ar}$ ,  $\text{He}$  and  $\text{H}_2$ , all of which could be expected to be present in volcanic ejecta in small amounts, could in principle survive such low temperatures and would provide most of the atmosphere in such situations. The argon could conceivably condense very close to the poles of Io and consequently have a very low average vapour pressure. This is not possible for hydrogen and helium. The evaporative and magnetospheric losses of these constituents must be severe enough to prevent them from becoming dominant. In fact, the ionisation time scale in the hot dense plasma which constitutes the magnetospheric environment of Io is sufficiently short (less than the orbital/rotation period of the satellite) that there is little chance for these constituents to accumulate. Similarly, secondary products such as  $\text{O}_2$ , which could be produced as a result of photolysis<sup>16</sup> or the effects of energetic magnetospheric particles, would also be lost rapidly.

We now consider the interaction between the magnetospheric plasma and magnetic field and the atmosphere of Io and in particular the abovementioned constraints on the interaction. The approximately exospheric (column content  $\leq 10^{15} \text{ cm}^2$ ) nature of the atmosphere shown in Fig. 2 allows for the direct escape of sputtered material over a large part of the surface. As pointed out by Johnson *et al.*<sup>2</sup> a relatively dense 'ionosphere' can be produced in such a rarified atmosphere if the ionisation is due to the presence of a flux of relatively soft ( $10\text{--}10^2$  eV) electrons, which are known to exist in the magnetospheric plasma torus (refs 3, 18, and H. E. Johnson and W. I. A. unpublished data). However, much of the ionosphere may not be as significantly affected by recombination as assumed by these authors because



**Fig. 1** The vapour pressures for various possible atmospheric constituents of Io as function of surface temperature. The curves are extrapolated from the data of refs 36, 37. The upper limit of the surface pressure determined by stellar occultation measurement is  $\approx 10^{-4}$  mbar (ref. 15); and the broken line marks the exospheric limit of  $3 \times 10^{-8}$  mbar. The surface temperatures at various latitudes ( $\theta$ ) with a  $(\cos \theta)^{1/4}$  dependence are marked in the bottom of the diagram.



**Fig. 2** The variations of  $\text{SO}_2$  vapour pressure on Io as a function of longitude from the subsolar point. The curve for  $x=0$  is approximated from the numerical results of Sinton<sup>38</sup> and the curves for  $x \neq 0$  are derived by assuming the surface material has thermal inertia  $1.3 \times 10^4 \text{ erg cm}^{-1} \text{ s}^{-1} \text{ K}^{-1}$ , heat capacity  $10^7 \text{ erg K}^{-1} \text{ g}^{-1}$ , a density of  $0.1 \text{ g cm}^{-3}$ , and thermal conductivity  $1.7 \times 10^2 \text{ erg cm}^{-1} \text{ K}^{-1} \text{ s}^{-1}$ . Differing values of  $x$  correspond to differing thicknesses of a fine dust layer which could conceivably exist on parts of the surface at least.

the convective removal time is shorter than the dissociative recombination time except in regions where the plasma density is  $\gg 10^5 \text{ cm}^{-3}$  or the atmosphere is sufficiently dense to prevent rapid motion of the plasma. In these circumstances, the plasma density is controlled by convection giving rise to the leading-trailing asymmetry observed at the limb<sup>3</sup>. Obviously, over the subsolar point where the atmospheric density is large, the structure of the ionosphere must be much more complex and depends on the orbital phase of Io.

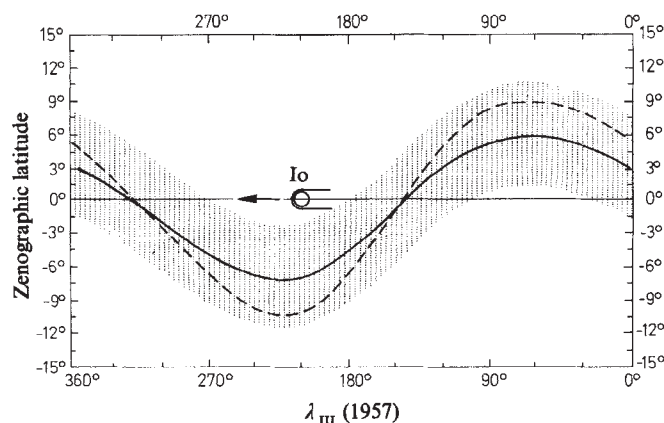
As the atmosphere of Io is mainly exospheric the interaction with the magnetosphere is correspondingly weak except in the vicinity of the subsolar point where the surface pressure is relatively large. This implies that the incident magnetic field lines to a large extent cross the satellite relatively freely and the energetic particles they carry with them can suffer absorption on the surface as required by the observations<sup>7,8</sup>. However, the interaction should be capable of producing a fairly large electric current ( $\sim 10^6\text{--}10^7$  A) to account for the Io-related decametric radio emissions. That is, a drag must be exerted on the corotating plasma and the magnetic field correspondingly stressed, so that a disturbance in the form of an Alfvén wave propagates in both directions along the field lines towards the planet<sup>4,5,19</sup>. This could be to a large extent the result of ion-neutral collisions (Pedersen conductivity) in the dense atmosphere surrounding the subsolar point, in which case a very strong longitudinal control of the radio emissions might be expected. However, note that the effect of ion mass pick-up, which has previously been overlooked in this connection (although alluded to in ref. 9)), could make a significant if not dominant contribution in this respect.

The effects of ion mass pick-up in the presence of a magnetic field have been considered in contexts other than the Io-magnetosphere interaction, notably the interaction between the solar wind and comets<sup>20,21</sup>, the ionospheres of Mars, Venus and Mercury<sup>22-25</sup> and the interstellar gas<sup>26,27</sup>. The processes involved can be understood using the mass and momentum conservation equations for the plasma which are good approximations in the circumstances of interest here:

$$\frac{dn}{dt} = q \quad (1)$$

$$\frac{d}{dt}(nmV) = nm \frac{dV}{dt} + mqV = j \times B \quad (2)$$

$$E + (V + B) = j \times B / ne \quad (3)$$



**Fig. 3** Geometrical relationship between the corotating thermal plasma disk and the orbital position of Io. The shaded region represents the sulphur ion nebula with thickness equal to one jovian radius<sup>32-34</sup>. The solid curve indicates the central plane of the plasma disk, and the broken line the magnetic equator according to the Goddard Space Flight Center  $O_4$  magnetic field model<sup>35</sup>. The direction of motion of Io in the rotating frame of the jovian magnetosphere and the convection pattern of the ionospheric plasma are also sketched. A current directed outward from Jupiter is generated in the extended atmosphere of Io to accelerate the newly created satellite ions into corotational motion. Io periodically crosses the plasma disk/magnetic equator at System III longitudes  $\lambda_{III} \approx 120^\circ$  and  $315^\circ$ .

$n$  is the plasma density,  $V$  is its velocity,  $m$  is the mean ion mass,  $q$  is the effective source strength due to ionisation of neutrals,  $E$  is the electric field,  $e$  the charge on an electron, and  $j$  and  $B$  are the electric current and magnetic induction respectively. As a first approximation, we neglect the effects of plasma pressure gradients and ion-neutral collisions and note that  $q$  must be proportional to the neutral density  $N$  regardless of the ionisation process. These equations, together with Maxwell's equations and suitable boundary conditions, determine the behaviour of the plasma. The two terms on the left of equation (2) can be regarded as sources of electric current, the first of which involves the deceleration and acceleration of the plasma (that is the polarisation current) and the second describes the effects of mass loading due to pick-up or loss of plasma by ionisation of neutrals and recombination or absorption, respectively. The latter term is negligible in most ionospheric situations but not in the case under consideration.

Taking  $q = \alpha N$  with  $\alpha$  and also  $V$  constant to a first approximation, we find from equation (1) that

$$\begin{aligned} n' &= (\alpha N_0 H / V) \exp(-z/H) \text{ upstream} \\ &= \alpha N_0 H / V (1 - \exp(-z/H)) \text{ downstream} \end{aligned} \quad (4)$$

where  $n'$  is the additional plasma density produced by ionisation of neutrals,  $z$  is the height above the surface of Io (for normal incidence), and  $N_0 H$  is the column content of the atmosphere. These solutions are, of course, highly idealised but they demonstrate certain basic features of the interaction, notably the upstream-downstream asymmetry and the relationship between the scale heights of the ionosphere and neutral atmosphere. To produce the additional plasma density of  $5 \times 10^3 \text{ cm}^{-3}$  observed on the upstream side of Io by Pioneer 10 (which, as it used a refraction technique, was unable to detect the uniform ambient magnetospheric plasma)  $\alpha$  must be  $2.5 \times 10^{-5} \text{ s}^{-1}$  for  $N_0 H \approx 10^{13} \text{ cm}^{-2}$ .

Clearly it is not easy to predict the form of the electric current distribution resulting from such an interaction as it requires a knowledge of the precise form of the flow field as well as the sources and sinks of plasma and if full three-dimensional treatment is warranted. However, one can estimate the magnitude of

a typical component of the current associated with mass pick-up (equation (2)) as simply

$$I = \dot{M}V/BL \quad (5)$$

Here  $\dot{M}$  is the total mass picked up by the magnetosphere and  $L$  is a length which is of the order of the diameter of Io. We estimate that if  $\dot{M} \approx 2.5 \times 10^5 \text{ g s}^{-1}$  on the upstream side of Io then with  $V = 57 \text{ km s}^{-1}$  and  $L = 4,000 \text{ km}$ , the corresponding electric current induced is  $I \approx 2 \times 10^6 \text{ A}$ . For the whole interaction, including the downstream region, the electric current may be significantly larger. The effective ionospheric conductance associated with ionisation of the exosphere is  $\Sigma = I/EL \approx 5 \text{ S}$  and the energy dissipation is  $IEL \approx 8 \times 10^{18} \text{ erg s}^{-1}$ . Note that the effects of the inertial term on the left hand side of equation (2) tend to reduce the current density on the upstream side of the satellite where the plasma decelerates by a factor of order  $(1 + X^2)^{1/2}$  where  $X = \omega L'/V$ ,  $L'$  is a characteristic length, and  $\omega$  is the gyrofrequency of the newly created ions in the ambient magnetic field. This reduction is significant on the upstream side as  $L' \approx H$  and  $X \approx 1$ , but there is no diminution of the current overall as all the freshly produced ions must be accelerated eventually up to the corotational speed of the magnetosphere relative to Io. It can be seen that equation (5) also applies to the Io torus of more extensive structure.

All aspects of the interaction between Io and the jovian magnetosphere can, therefore, be accounted for if the atmosphere has the properties indicated in Fig. 2 and we suggest that ion mass pick-up is an important ingredient of this interaction. These arguments lead to some interesting conclusions on the variation of the nature of the interaction with both the jovian longitude and the Io phase. As the dense regions of the atmosphere influence the interaction the phase of Io in its orbit must be important as, for example, the subsolar point is on the leading-trailing side of the satellite at western-eastern elongations respectively. Also the fact that the jovian plasma sheet is not coplanar with the orbit of Io (see Fig. 3) should lead to a jovian longitudinal control of the currents produced by the interaction if the total ionisation rate is the determining factor and this is controlled by the density of the corotating magnetospheric plasma. Note that the plasma torus need not instantaneously coincide with the equilibrium position shown since it can oscillate in position along the magnetic field with a frequency  $\sqrt{3}\Omega/2\pi$ , where  $2\pi/\Omega$  is the rotational period of Jupiter. This is not commensurate with the (relative) orbital period of Io and hence the addition of fresh plasma with random phase will tend to suppress the oscillations. Nevertheless, the oscillations should be completely absent only at longitudes  $\lambda_{III} \approx 120^\circ$  and  $350^\circ$  where Io crosses the jovian magnetic equator.

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Note that several of our conclusions have been reached independently in papers submitted later than our original manuscripts. In particular, we refer to the discovery of  $\text{SO}_2$  gas on Io by Pearl *et al.*<sup>28</sup> and the identification of  $\text{SO}_2$  frost on the surface by Fanale *et al.*<sup>29</sup>. Smith *et al.*<sup>30</sup>, Pearl *et al.*<sup>28</sup> and Kumar<sup>31</sup> have also drawn attention to the fact that the atmosphere of Io should be spatially inhomogeneous.

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## Accretion of nitrogen during the growth of planets

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The  $^{15}\text{N}/^{14}\text{N}$  ratio measured in nitrogen of the terrestrial atmosphere is, or was, apparently widespread in the inner Solar System. Such an observation might suggest that this value ( $3.61 \times 10^{-3}$ ) represents that of proto-solar material. However, I argue here that the original ratio was at least 20% lower, the presently observed value having resulted from significant isotopic fractionation, presumably caused by nebular condensation processes.

First, I will summarise what we know about  $^{15}\text{N}/^{14}\text{N}$  of inner Solar System objects, expressing these values as per cent deviations from the terrestrial atmospheric value. The Moon is highly depleted in volatiles, and identification of indigenous lunar N, free from either terrestrial contamination or cosmic ray spallation products, has consequently been very difficult. The best estimate for the lunar  $^{15}\text{N}/^{14}\text{N}$  ratio is between +1 and +4% relative to air<sup>1</sup>.

The martian atmosphere is highly enriched in  $^{15}\text{N}$ , about +62% relative to air<sup>2</sup>. However, it has been argued that this value has resulted from selective escape of  $^{14}\text{N}$  from the martian upper atmosphere<sup>2</sup>. When allowance is made for this fractionation, a starting composition close to the terrestrial value is derived<sup>2</sup>. Because of the model dependence of the fractionation calculations, it is not possible to assign realistic uncertainty limits to the initial  $^{15}\text{N}/^{14}\text{N}$  ratio. A large uncertainty is also associated with the  $^{15}\text{N}/^{14}\text{N}$  ratio found for the atmosphere of Venus. The value measured by Pioneer Venus is within 20% of that of terrestrial air<sup>3</sup>.

Different groups of stony meteorites have significantly different N isotopic compositions<sup>4,5</sup>, although the actual nature of the relationship between  $^{15}\text{N}/^{14}\text{N}$  ratio and chemical-petrographic classification is unclear. Referring to Fig. 1 of ref. 5, ordinary chondrites and carbonaceous chondrites of petrologic types 3 and 4 lie close to the terrestrial value (−0.3 to +2.0% and −4.3 to +0.2%, respectively). Carbonaceous chondrites of petrologic types 1 and 2 are enriched in  $^{15}\text{N}$  by up to 4.6% (with one large anomaly, Renazzo, at +17.2%), the degree of enrichment roughly correlating with increasing N content<sup>5</sup>. Enstatite chondrites, on the other hand, are depleted in  $^{15}\text{N}$  (to −4.3%), with a rough tendency for  $^{15}\text{N}/^{14}\text{N}$  to decrease with increasing N content<sup>5</sup>. Whatever the cause(s) of these isotopic patterns, the trend of the meteoritic data suggests that the present compositions reflect fractionations, in different directions, of a starting composition close to that of the terrestrial

atmosphere. Apparently, formation of organic matter in carbonaceous chondrites and inorganic nitrides in enstatite chondrites favoured heavy and light isotopes, respectively.

This picture of isotopic uniformity in the inner Solar System is disturbed when we turn to the only other repository of N analysed so far: the solar wind, believed to represent the composition of the solar convective zone<sup>6</sup>. These analyses have been made using solar wind ions implanted in the lunar surface during the past 4 Gyr, and have revealed that  $^{15}\text{N}/^{14}\text{N}$  in the Sun has increased substantially over the lifetime of the Moon<sup>7</sup>. The actual magnitude of the change is still not established precisely but is known to be at least 30%, from −19% (ref. 8) to +11% (ref. 1) relative to air. Whatever the cause of this change, possibly nuclear reactions in the solar convective zone<sup>7</sup>, it seems clear that the solar atmosphere must have had an initial  $^{15}\text{N}/^{14}\text{N}$  ratio at least 19% lower than terrestrial air. It is therefore difficult to avoid the conclusion that this lower value must also have characterised the proto-solar nebula. This in turn leads to the conclusion that the value observed or inferred for the Earth and other planetary objects must represent the result of significant isotopic fractionation.

It is unlikely that we can specify, at this time, how such fractionation occurred. The fact that its magnitude was apparently independent of either the size of the planetary object (radii varying from some tens of kilometres<sup>9</sup> to more than 6,000 km) or its degree of volatile depletion (from about  $10^{-7}$  to  $10^{-3}$  g N per g) suggests that N, and presumably other volatiles, were accreted by early forming planets, and planetesimals, in the form of a solid component of volatile-rich condensate. Isotopic fractionation apparently took place between this component and the reservoir from which it condensed, rather than during accretion. The magnitude and sign of the fractionation suggest gas–solid equilibration at very low temperature rather than kinetic effects or isotopically selective loss of gas.

Because neither the nature of the N-rich condensate nor its temperature of formation is known, it is not possible to determine whether the fractionation factor associated with the condensation reaction(s) is adequate to provide the degree of fractionation implied here. Calculated fractionation factors<sup>10</sup> for isotopic exchange between simple N compounds imply an equilibration temperature significantly below 273 K. Equilibration at such low temperatures in the nebula seems intuitively unlikely but similar temperatures are suggested by the enrichment of the Earth and carbonaceous chondrites in deuterium, relative to its abundance in the primitive solar nebula<sup>11–13</sup>.

The condensation/accretion scenario suggested here is qualitatively consistent with the model of Anders and Owen<sup>14</sup>, which proposes acquisition of the Earth's volatiles via a veneer of composition similar to that of carbonaceous chondrites of petrologic type 3, which also provide a reasonable match in  $^{15}\text{N}/^{14}\text{N}$  ratios<sup>5</sup>. However, the magnitude of the fractionation suggested here, between the volatile-carrier and its parental reservoir, implies a temperature of formation significantly lower than the 400–450 K inferred by Anders and Owen for carbonaceous chondrite material<sup>14</sup>.

The hypothesis presented here is potentially testable as it leads to three observational predictions. First, the  $^{15}\text{N}/^{14}\text{N}$  ratio in Jupiter, which should directly reflect that of proto-solar material, will be found to be at least 19% lower than terrestrial air. Second, the  $^{15}\text{N}/^{14}\text{N}$  ratio in the present solar wind will be found to be about 11% greater than in air (more if we do not measure it soon). Third, isotopic fractionation of N during condensation may have been accompanied by similar fractionation of any other volatile elements condensing at the same time. If such elements, or the appropriate fractions of them, can be isolated for analysis, similar heavy isotope enrichments may be detected. In fact, the deuterium mentioned above may constitute such evidence; however, the relationship between the provenance of nitrogen and that of hydrogen in early Solar System objects remains a topic for further study.

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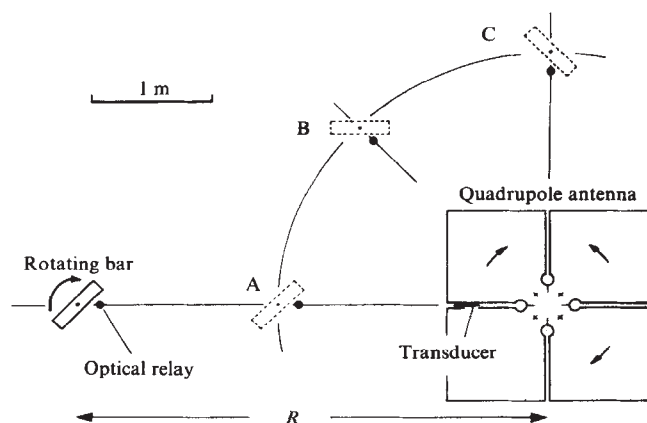
## Dynamical test of the law of gravitation

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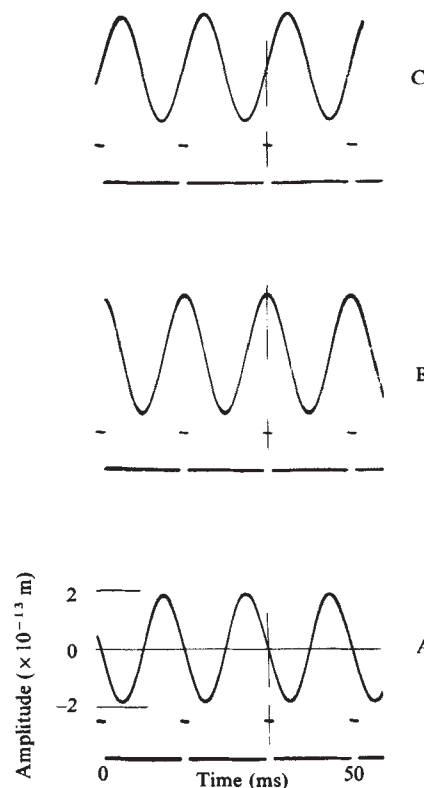
Although well established in the realm of celestial mechanics, gravitation between two masses has not yet been thoroughly examined in a laboratory. There have been several tests of the interaction since the Cavendish's celebrated experiment<sup>1-6</sup>, most of them measuring the static attraction between two spherical masses at a distance  $R$  less than 0.8 m. The gravitational interaction is weak and, in contrast with the electromagnetic interaction, there is no effective means to shield it. It has therefore proved difficult to increase the distance  $R$  over 1 m. Under the circumstances several authors<sup>7-9</sup> have noted that the existing experimental data do not exclude a possible term which is added to the newtonian  $1/R$  potential. Meanwhile, on the grounds of elementary particle physics, a number of theoretical papers<sup>7,10-14</sup> have been published predicting additional terms which vanish rapidly at  $R = \infty$ . We now report a test of the law of gravitation in a range of  $R$  up to 4.2 m. The experiment is based on a low frequency dynamical quadrupole-quadrupole interaction between two masses, which was first observed by Sinsky and Weber<sup>15,16</sup>. Our result agrees with the inverse square law within the accuracy of the experiment.

A steel bar of length  $b = 0.52$  m and mass  $m = 44$  kg was rotated in a horizontal plane at frequency  $f = 30.3$  Hz and the resulting dynamic gravitational field was measured by a mass quadrupole antenna<sup>17</sup> resonating at  $2f = 60.5$  Hz (Fig. 1). The antenna is a 1,400-kg aluminium square plate with cuts on each side, contained in a vacuum tank of a constant temperature,



**Fig. 1** Dynamic gravitational field of a 44-kg steel bar rotating at 30.3 Hz was measured by a 1,400-kg aluminium quadrupole antenna resonating at 60.5 Hz. Signals were obtained for a distance  $R$  up to 4.2 m.

$T = 308$  K. The oscillation of the antenna was sensed by an electrostatic transducer of field strength  $4.6 \times 10^6$  V m<sup>-1</sup> mounted inside one of the cuts. The quality factor  $Q$  of the unloaded antenna was 65,000 at 60.5 Hz. The resulting width of the resonance,  $9.3 \times 10^{-4}$  Hz, was inconveniently narrow for a long-term operation requiring a precise frequency tracking. Therefore we applied a cold damping<sup>18</sup> to the antenna, loading it with an artificial cold resistor<sup>19</sup>, 6.3 M $\Omega$  at 3.0 K. This resulted in a reduced quality factor  $Q' = 4,100$ , a resonance width  $1.5 \times 10^{-2}$  Hz, and a noise temperature  $T' = 22$  K of the antenna. With the rotor located at  $R = 2.2$  m, the antenna oscillated with an amplitude  $x = 1.9 \times 10^{-13}$  m in regard to the transducer displacement. This yielded a coherent  $8.7 \times 10^{-7}$  V (peak) transducer output, while the background noise, consisting mostly of

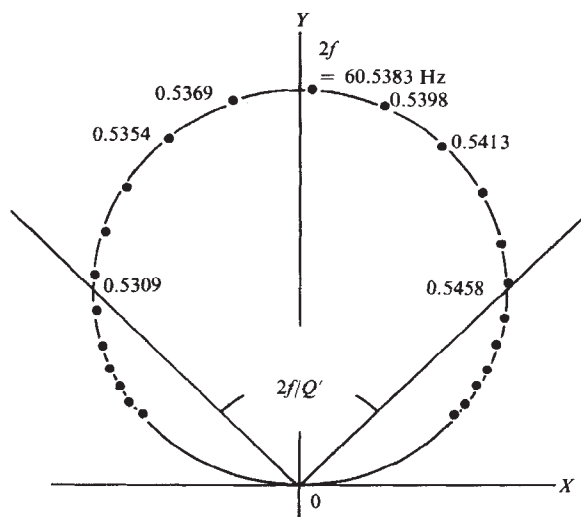


**Fig. 2** Oscillation of the antenna observed on a cathode-ray tube with the rotor located at A, B and C of Fig. 1 respectively. Distance  $R = 2.2$  m. The timing pulses obtained from an optical relay indicate the rotation angle of the bar. Note the difference in the phase of oscillation in three pictures. The signal at B is slightly larger than the others, showing the effect of the higher mass multiple moments of the antenna. With an increasing distance  $R$ , the ratio of the signal B to signal A (=C) tends to 16/19.

the antenna brownian motion at  $T' = 22$  K, was  $1.2 \times 10^{-8}$  V (r.m.s.).

To eliminate a possible interaction of the antenna with coherent noises in the background, we rotated the bar in a steel vacuum tank using a plastic chain and two sprocket wheels of a teeth ratio 8/5. The larger wheel was mounted on a common shaft of a 400-W induction motor and a phase-locking 200-W synchronous motor, the latter being driven by a 37.8-Hz three-phase power supply connected to a quartz frequency synthesiser. In spite of these measures, we noted a coherent magnetic field around the tank,  $10^{-4}$  G at 60.5 Hz and a smaller amount at 30.3 Hz, presumably originating from a geomagnetic or remanent magnetisation of the rotating steel bar. We checked, however, that a magnetic field, 5 G at 60.5 Hz, more than 90 db above the background was needed to produce a





**Fig. 3** Characteristics of the antenna resonance at 60.5 Hz. The amplitude and phase of the forced vibration of the antenna is given as a function of the frequency  $2f$  of the oscillating field. Quality factor of the antenna was reduced to  $Q' = 4,100$  by application of a cold damping.

motion of a damped linear oscillator quite well. The amplitude of vibration is given by

$$x = \frac{\gamma GaQ'mb^2}{R^5 f^2}$$

where  $a$  is the size of the antenna.  $\gamma$  is a numerical coefficient which depends on the geometry of experiment including the shape, the size, and the relative orientation of the masses. Figure 2 shows the antenna output signal obtained with the rotor at three locations around the antenna. The timing signal indicating the angle of the bar is also shown. The characteristics of the antenna resonance are given by a circular locus of Fig. 3 showing the observed amplitude and phase of vibration as a function of the frequency  $2f$  of the oscillating field. The signal decreases with increasing distance  $R$  as  $1/R^5$ . However the signal-to-noise ratio can be improved substantially by means of Fourier integration<sup>20</sup> of the antenna output with the rotor timing signal as a reference. Thus we obtained a signal at  $R = 4.2$  m, 30 db above the brownian motion, with an integration time of 4 h. Figure 4 compares the observed signal and the expected signal based on the inverse square law, with the distance  $R$  as a parameter. This result confirms Newton's law of gravitation within the range and accuracy,  $\pm 3\%$ , of the experiment. For a parameter  $\delta$  in an assumed form,  $1/R^{1+\delta}$ , of the potential, we have obtained  $0.000 \pm 0.053$ .

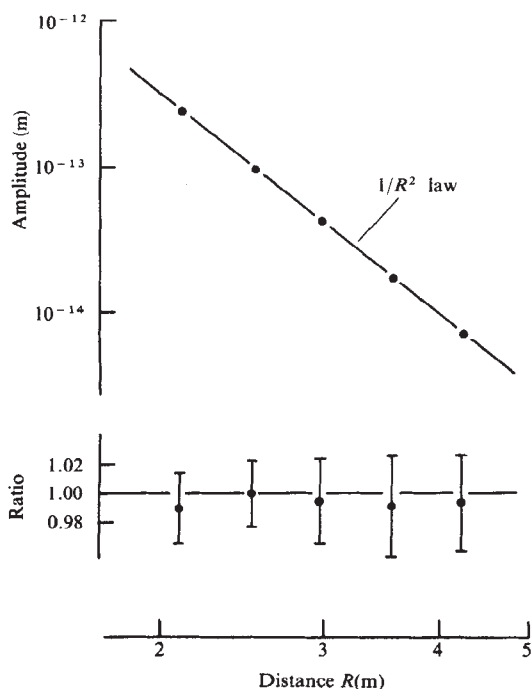
We thank Mr H. Yoshikawa for construction of the rotor. This work was supported by a grant from the Mitsubishi Foundation.

spurious antenna output of the same size as the observed signal. The rotor also caused vibration of the laboratory floor at 60.5 Hz. Two decks of pneumatic isolation pads were inserted below the rotor tank. They were more than enough to eliminate the contamination of the signal through this channel. In fact no change of signal was noted when one of the decks was removed.

The behaviour of the mass quadrupole antenna in the gravitational field of the rotating bar obeys an equation of forced

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**Fig. 4** The observed and calculated intensities of the signal and their ratio as a function of the distance  $R$ . The scale of the observed signal is normalised to the calculated signal at  $R = 2.5$  m. The calculation is based on the inverse square law of gravitation. The effect of the higher multipole moments, the finite size of the rotor and the antenna, is taken into account by a numerical integration over the masses.

## Stress corrosion cracking of biotite and feldspar

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Pervasive intragranular cracking of biotite and feldspar has been observed in a naturally deformed granodiorite, where the leading edge of cracks are sites for specific reactions of parent grains to secondary mineral products. This textural feature provides evidence for an interrelationship of chemical reactions to slow crack propagation, and is considered to be a natural example of stress corrosion cracking. It is probable that the mineral reactions are catalysed by increase of the stress intensity at fracture tips, with reaction rates controlling propagation of the fractures.

**Table 1** Chemical analyses of a biotite grain with cracks containing secondary minerals

|                                | 1                   | 2            | 3            | 4            | 5            | 6            | 7            | 8            |
|--------------------------------|---------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| SiO <sub>2</sub>               | 35.64 (0.22)        | 35.27        | 35.16        | 35.32        | 35.88        | 44.83        | 48.57        | 35.07        |
| Al <sub>2</sub> O <sub>3</sub> | 18.19 (0.18)        | 18.09        | 18.33        | 18.52        | 18.41        | 30.91        | 34.32        | 18.46        |
| TiO <sub>2</sub>               | 4.19 (0.14)         | 3.73         | 3.39         | 3.07         | 2.66         | 3.16         | 1.36         | 1.63         |
| FeO                            | 20.85 (0.31)        | 21.19        | 21.49        | 21.13        | 21.71        | 5.21         | 0.91         | 23.51        |
| MgO                            | 7.14 (0.45)         | 7.04         | 7.00         | 7.38         | 7.85         | 1.89         | 0.41         | 7.73         |
| MnO                            | 0.21 (0.07)         | 0.16         | 0.29         | 0.16         | 0.20         | —            | —            | 0.19         |
| CaO                            | —                   | —            | —            | —            | 0.05         | —            | —            | 0.05         |
| Na <sub>2</sub> O              | 0.13 (0.04)         | 0.13         | 0.07         | 0.04         | 0.10         | 0.18         | 0.14         | 0.04         |
| K <sub>2</sub> O               | 9.50 (0.18)         | 9.76         | 9.63         | 9.72         | 9.47         | 9.63         | 8.75         | 9.21         |
|                                | <u>95.87</u> (0.54) | <u>95.37</u> | <u>95.41</u> | <u>95.34</u> | <u>96.33</u> | <u>95.81</u> | <u>94.46</u> | <u>95.89</u> |
| Si                             | 5.437 (0.047)       | 5.428        | 5.419        | 5.426        | 5.461        | 6.067        | 6.400        | 5.409        |
| Al(IV)                         | 2.563 (0.047)       | 2.572        | 2.581        | 2.574        | 2.539        | 1.933        | 1.600        | 2.591        |
| Σ                              | 8.000               | 8.000        | 8.000        | 8.000        | 8.000        | 8.000        | 8.000        | 8.000        |
| Al(VI)                         | 0.707 (0.050)       | 0.709        | 0.747        | 0.779        | 0.764        | 2.996        | 3.729        | 0.765        |
| Ti                             | 0.481 (0.014)       | 0.432        | 0.393        | 0.355        | 0.304        | 0.322        | 0.135        | 0.189        |
| Fe                             | 2.660 (0.047)       | 2.727        | 2.770        | 2.715        | 2.764        | 0.590        | 0.100        | 3.033        |
| Mg                             | 1.622 (0.095)       | 1.615        | 1.608        | 1.690        | 1.781        | 0.381        | 0.081        | 1.777        |
| Mn                             | 0.031 (0.011)       | 0.021        | 0.038        | 0.021        | 0.021        | —            | —            | 0.025        |
| Σ                              | <u>5.501</u>        | <u>5.504</u> | <u>5.556</u> | <u>5.558</u> | <u>5.638</u> | <u>4.289</u> | <u>4.044</u> | <u>5.786</u> |
| Ca                             | —                   | —            | —            | —            | 0.008        | —            | —            | 0.008        |
| Na                             | 0.039 (0.013)       | 0.039        | 0.021        | 0.012        | 0.030        | 0.047        | 0.036        | 0.012        |
| K                              | 1.848 (0.032)       | 1.916        | 1.893        | 1.905        | 1.839        | 1.662        | 1.471        | 1.812        |
| Σ                              | <u>1.887</u>        | <u>1.955</u> | <u>1.914</u> | <u>1.916</u> | <u>1.876</u> | <u>1.710</u> | <u>1.506</u> | <u>1.832</u> |

(1) Average of five analyses from the centre of a primary biotite grain. Figures in parentheses represent one standard deviation. (2) Biotite parent grain 200 µm from crack. (3) Biotite parent grain in pale domain 15 µm from crack. (4), (5) Biotite parent grain at leading edge of crack. (6), (7) Secondary high-Ti muscovite coexisting with secondary ilmenite in crack. (8) Secondary biotite recrystallised from parent at margin of grain.

**Table 2** Chemical analyses of orthoclase and plagioclase grains with cracks containing secondary feldspar

|                                | 1                     | 2             | 3             | 4             | 5             | 6             | 7             | 8             | 9             | 10            | 11            |
|--------------------------------|-----------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| SiO <sub>2</sub>               | 65.05 (0.38)          | 64.92         | 68.19         | 64.97         | 68.25         | 62.29         | 65.04         | 61.41         | 64.81         | 69.18         | 66.07         |
| Al <sub>2</sub> O <sub>3</sub> | 17.04 (0.31)          | 18.09         | 20.77         | 18.33         | 19.55         | 23.60         | 18.45         | 23.10         | 23.56         | 22.53         | 17.91         |
| CaO                            | 0.06 (0.03)           | 0.15          | 0.66          | 0.02          | 0.77          | 5.48          | 0.03          | 4.92          | 1.97          | 1.77          | 0.07          |
| Na <sub>2</sub> O              | 0.45 (0.03)           | 0.39          | 10.45         | 0.38          | 11.16         | 8.39          | 0.44          | 6.22          | 7.41          | 6.54          | 0.48          |
| K <sub>2</sub> O               | 16.43 (0.11)          | 16.32         | 0.37          | 16.58         | 0.14          | 0.59          | 16.64         | 2.51          | 2.15          | 1.38          | 15.98         |
| Total                          | <u>99.05</u> (0.66)   | <u>99.87</u>  | <u>100.43</u> | <u>100.07</u> | <u>99.87</u>  | <u>100.34</u> | <u>100.60</u> | <u>98.17</u>  | <u>99.90</u>  | <u>101.39</u> | <u>100.52</u> |
| Si                             | 12.152 (0.041)        | 12.020        | 11.785        | 12.020        | 11.883        | 11.031        | 11.977        | 11.074        | 11.398        | 11.820        | 12.113        |
| Al                             | 3.752 (0.056)         | 3.949         | 4.230         | 3.953         | 4.012         | 4.926         | 4.004         | 4.992         | 4.883         | 4.535         | 3.867         |
| Na                             | 0.164 (0.010)         | 0.141         | 3.836         | 0.137         | 4.105         | 2.879         | 0.156         | 2.211         | 2.527         | 2.168         | 0.172         |
| Ca                             | 0.014 (0.008)         | 0.031         | 0.121         | 0.004         | 0.145         | 1.039         | 0.004         | 0.965         | 0.371         | 0.324         | 0.016         |
| K                              | 3.917 (0.002)         | 3.855         | 0.082         | 3.914         | 0.031         | 0.133         | 3.910         | 0.586         | 0.484         | 0.301         | 3.738         |
| Σ                              | <u>20.000</u> (0.010) | <u>19.996</u> | <u>20.055</u> | <u>20.027</u> | <u>20.176</u> | <u>20.008</u> | <u>20.051</u> | <u>19.828</u> | <u>19.664</u> | <u>19.148</u> | <u>19.906</u> |

(1) Orthoclase parent grain with fractures containing secondary fibrous orthoclase (2). Values in parentheses represent one standard deviation. (3) Albite parent grain with both secondary orthoclase (4) and albite (5) fracture fillings. (6) Oligoclase parent grain with secondary orthoclase (7) in fractures. (8) Parent oligoclase grain with abundant fine grained sericite, recrystallising to a less calcic, sericite-free plagioclase at the leading edge of (10) and within fractures (9). Also present in the oligoclase parent grains are fractures containing secondary orthoclase (11).

The process of stress corrosion cracking has been reviewed by Anderson and Grew<sup>1</sup>. Slow crack growth at subcritical stress intensities by stress corrosion contrasts with fast irreversible propagation of 'Griffith cracks' above the critical stress value for brittle failure. Subcritical crack growth has been extensively studied in metals and ceramics<sup>1-4</sup>. Time-dependent cracking of quartz and granite has been experimentally demonstrated by Martin<sup>5</sup> and Wilkins<sup>6</sup> respectively, and slow growth of microfractures implicated in a number of geological phenomena such as pre-faulting dilatancy and magmatic intrusion<sup>1</sup>.

However, we are not aware of any previous descriptions of chemical reactions accompanying cracking in naturally deformed rocks, or an identification of the reactions involved. The

rocks to be described which exhibit intragranular fracturing accompanied by mineral reactions are located at the boundary of a major subvertical shear zone which transects granodiorite basement of the Aiguilles Rouge Massif, at Miéville, Switzerland. The granodiorite consists of orthoclase and plagioclase (~An<sub>25</sub>-An<sub>18</sub>) feldspar phenocrysts (45-60%), quartz (25-30%) and biotite (10-30%) as essential minerals. At increasing states of deformation the primary minerals are progressively reduced in grain size by a factor of 100-1,000 through microfracturing and recrystallisation. Accompanying the deformation is production of secondary albite, chlorite, epidote, calcite, ilmenite and sphene. The ambient temperature of cracking is estimated to be <250 °C from (1) the presence of prehnite-



pumpellyite facies cover rocks to the basement, which are involved in the shear zone<sup>7</sup>, and (2) filling temperatures of 180–210 °C determined on primary fluid inclusions in feldspar fracture fillings. These represent minimum values inasmuch as corrections to temperature cannot be applied without precise knowledge of the hydrostatic pressure and fluid composition. However, corrections are probably less than +40 °C.

In rocks at a low state of deformation grains of biotite have intragranular fractures with a linear to sigmoidal shape which are bounded by an envelope of pale brown birefringence. This region corresponds to a decrease of Ti and increase of Fe relative to the composition of grains in areas unaffected by cracking. Fracture openings are occupied by secondary minerals, predominantly ilmenite, low-Ti biotite and high-Ti muscovite (Table 1). The presence of titanium in muscovite may be due to ultrafine grained disseminated ilmenite. These mineral products were also observed in kink bands, and at grain boundaries where the parent biotites have recrystallised, but are not present at grain boundaries in undeformed rocks. Parent grains and secondary products were examined using electron microprobe. Randomly orientated ilmenite grains (~10 µm long) are present up to 200 µm ahead of the leading edge of fractures (Fig. 1a). This region is characterised by the initiation of mineral reactions, and represents the first stage of chemically controlled disruption of the biotite structure where increase of the stress intensity at fracture tips may enhance lattice diffusion and thus catalyse reaction of the primary biotite. Decrease of volume

accompanying the reaction of biotite to ilmenite plus muscovite in cracks may also serve to increase the local stress intensity at fracture tips, and enhance permeability.

Localisation of reactions ahead of fracture tips may be a diagnostic criterion for stress corrosion cracking, in contrast to alteration of minerals along fractures by means of the enhanced access of reactants. The reactions accompanying cracking described above do not involve any net hydration, as indicated by the relatively constant proportion of volatiles in rocks at all observed states of deformation, and from considerations of the structural water content of reactants and products in cracks. In addition, the  $\delta^{18}\text{O}$  whole rock and ratio of  $\text{Fe}^{2+}/\Sigma\text{Fe}$ , both of which are sensitive to water-rock interaction, maintain constant values at increasing states of strain, implying no significant transport of fluids through the deforming rocks. However, the participation of an aqueous phase (as evidenced by the small quantities of fluids present in inclusions) is probably an important factor in catalysing reactions and the transport of components at fracture tips.

Grains of both orthoclase and plagioclase feldspar exhibit abundant microfractures of two distinct morphologies. First, linear fractures which terminate at grain boundaries, are narrow and involve transverse displacements of up to 3 mm (Fig. 1b). Second, irregular fractures which generally terminate within crystals, are relatively wide (20–200 µm), and have little discernible evidence of translation (Fig. 1b). The latter type of fractures are occupied by secondary reaction products of the parent grain, and are believed to have propagated by stress corrosion. Towards the boundaries and leading edge of irregular fractures parent grains become free of solid inclusions such as sericite, compared to regions remote from fracturing. During the cracking process sericite appears either to have migrated to the crack boundaries which it locally decorates, or to have reacted to secondary orthoclase in fractures, or to have been removed from the local chemical system. Secondary feldspar within fractures is clear, and contains abundant primary fluid inclusions in the sub-20 µm size range.

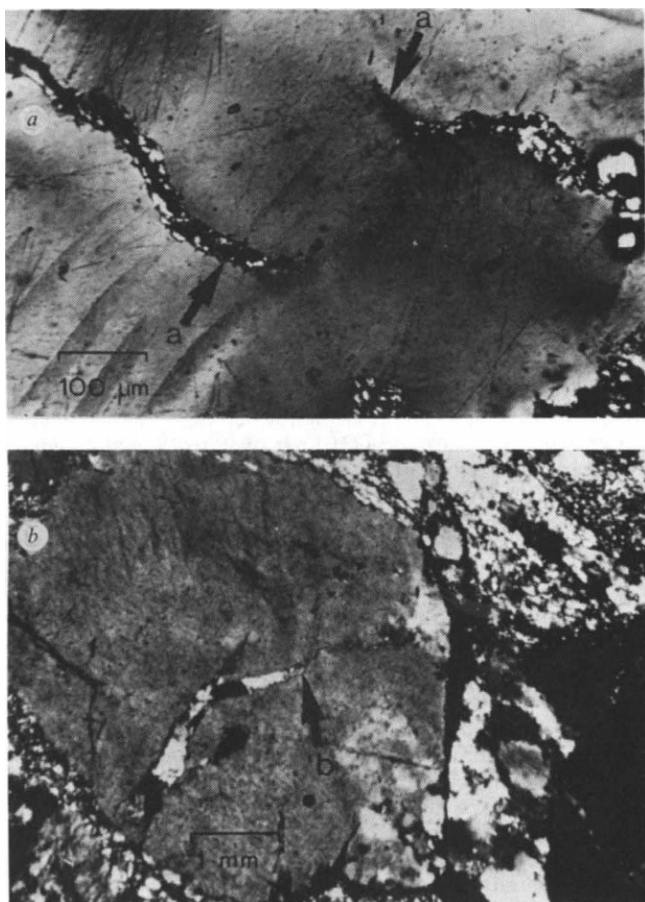
Dilatant fractures orientated normal to the local stretching direction are generally occupied by feldspar fibres growing in the direction of extension, bridging the fracture boundaries. In general, fractures are occupied with secondary feldspar of approximately the parent grain composition. However, many oligoclase phenocrysts recrystallise to a more sodic composition with corresponding loss of Ca, accompanied by loss of K as sericite is removed (Table 2). This reaction also proceeds during recrystallisation of oligoclase at grain boundaries<sup>7</sup>. Phenocrysts of orthoclase may have fracture fillings of orthoclase, plagioclase, or both feldspars coexisting, and this is also true for plagioclase phenocrysts (Table 2).

Stress corrosion cracking gives rise to grain size reduction in the rocks described and is probably operative in low to medium temperature environments where crystal plasticity is not generally predominant. However, its overall role in crustal deformation is not known. One possible consequence of this process is enhanced permeability, especially for reactions with a negative  $\Delta V$ .

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**Fig. 1** a, Basal plate of biotite, within deformed granodiorite, containing fractures occupied by secondary ilmenite (a), muscovite and biotite. b, Phenocryst of orthoclase in a deformed granodiorite containing irregular non-displacive tensile fractures (b) occupied by secondary orthoclase, and/or albite; bounded on right hand margin by linear fracture with displacement.

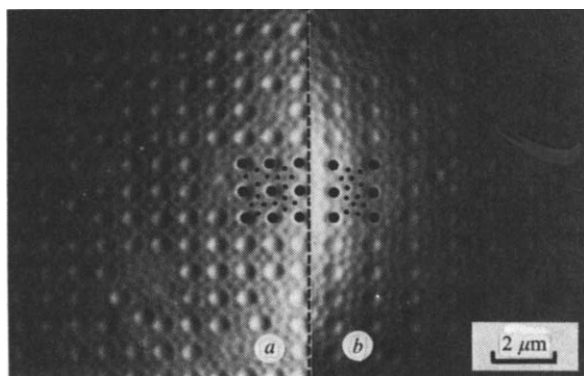
# Optical demonstration of crystalline superstructures in binary mixtures of latex globules

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The colloid known as monodisperse latex<sup>1</sup> is an aqueous suspension of a synthetic polymer, the particles of which are ideally spherical and uniform in size (monodisperse). It shows phase transition<sup>2</sup> from a disordered to an ordered state, changing its appearance from milky white to iridescent. This is considered as experimental verification of the transition found by computer calculations<sup>3,4</sup> of concentrated hard core systems. Thus, monodisperse latex is a reliable model for studying the statistical mechanics of concentrated systems. Furthermore, as the particles are visible<sup>5,6</sup> by light microscopy when they are larger than 0.3  $\mu\text{m}$ , we can now directly see what happens during phase transition and related phenomena. Recent work on the structure of gem opals<sup>7</sup> offers an important clue to understanding the statistical properties of binary systems. It showed that in some unusual opals two kinds of silica spheres of different size form a superstructure with long period. The structure must have been formed in a stable dispersion of silica spheres<sup>7</sup> and then been condensed and dehydrated. It should be possible to produce the structure artificially in some colloids and so provide an excellent model of binary systems. We report here that we have succeeded in producing superstructures in mixtures of two monodisperse polystyrene latexes of different particle size and have observed them microscopically<sup>5</sup> in their natural (stably dispersed) state. Several structures were found, including the alloy structures of  $\text{NaZn}_{13}$  and  $\text{CaCu}_5$  types, which belong to the so-called size-factor compounds<sup>8</sup>.

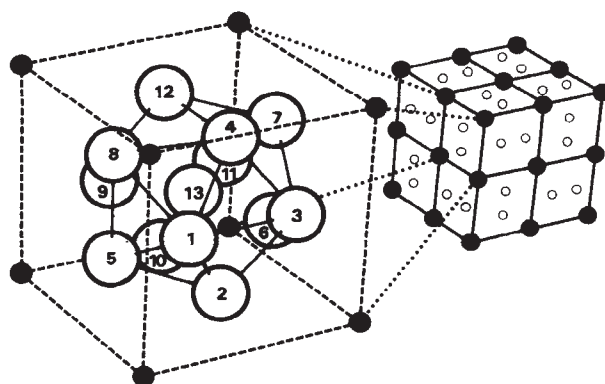
The survey was made over a wide range of particle-number ratio for many combinations of latexes. For every mixture, the total solid content was adjusted to about 0.1 volume fraction, and their electrolyte (KCl) concentration was maintained at around  $10^{-5} \text{ mol l}^{-1}$ .



**Fig. 1** Photomicrographs of the superstructure in a mixture of 5,500 Å and 2,700 Å latexes taken by light (metallurgical) microscope. Large spheres represent 5,500 Å particles and small ones 2,700 Å particles. Lattice type is simple cubic with respect to 5,500 Å particles. *a* Represents the (100) plane, and *b* the (110) plane. To distinguish the configuration of 2,700 Å particles, a superlattice is drawn. Staggered rhombuses can be seen in the (100) plane and a distorted pentagon in the (110) plane. The 2,700 Å particles were not static; from time to time they underwent a collective motion.

When conditions were optimal, in ten to several tens of hours after placing the cell on the microscope the ordered structures appeared. At this stage, the ordered regions were 'islands' about 50  $\mu\text{m}$  in diameter surrounded by a 'sea' of irregular structure, but within a few days had grown to reach an equilibrium state in which the ordered structures coexisted with the disordered structure. These features were in accord with Sanders' observations on opal specimens<sup>7</sup>.

The formation was quite reversible; the structures once formed could be broken by slight mechanical shock and reformed in several hours. The phenomenon resembled a kind of phase separation. The condition necessary for the ordered structures to appear is the basic problem of statistical mechanics of binary systems. It was rather strict, depending on the total solid content, the size ratio, the particle-number ratio and electrolyte concentration, and will not be discussed here. We will describe only two of the various structures discovered.



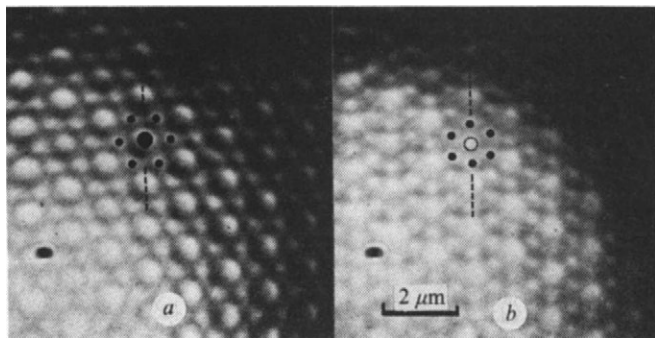
**Fig. 2** Superstructure of the  $\text{AB}_{13}$ -type structure. Small solid circles indicate the position of the 5,500 Å particles and open circles represent the 2,700 Å particles forming a centred icosahedron. Particles 1, 2, 3 and 4 appear under the microscope as a rhombus in the (100) plane. Particles 2, 3, 4, 8 and 5 form a distorted pentagon in the (110) plane. The orientation of the icosahedron changes by  $90^\circ$  between adjacent cells.

Figure 1 shows patterns that appeared in the 5,500–2,700 Å system, the particle-number ratio of which was 1 : 8. These are, respectively, the (100) and (110) net planes of a simple cubic lattice with respect to 5,500 Å particles. The lattice constant was 0.85  $\mu\text{m}$ . By moving the microscope focus upwards (an inverted-type microscope was used), we could find the next net plane. The mode of superposition of successive net planes and the measured spacing between them supported the simple cubic model.

The arrangement of the smaller particles is, however, rather complicated. The (100) plane contains four particles, which seem to be in the form of a rhombus (strictly speaking, it would be a shallow tetrahedron). The (110) plane contains six particles, five of which form a distorted pentagon with one particle at its centre. The centering particle must be situated at the body centre of the simple cubic lattice of 5,500 Å particles. Then, the simple cubic unit cell must contain four small particles in each of its side planes and one at its centre. This leads to the composition  $\text{AB}_{13}$  for this structure, in agreement with Sanders' opinion (personal communication).

The staggered arrangement of the rhombuses in the (100) plane indicates that the unit cell of the superstructure must be a block consisting of eight of the single cubic cells of 5,500 Å particles. The orientational behaviour of the distorted pentagon in the (110) plane is also interesting. These features are explained by assuming that the structure is as shown in Fig. 2. Note that this is the same, at least in particle configuration, as the structure of intermetallic compounds of  $\text{NaZn}_{13}$  type<sup>9</sup>.

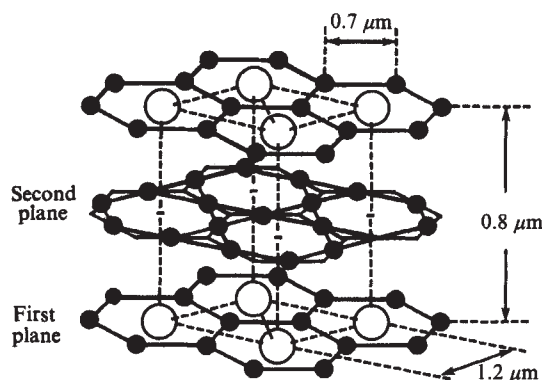




**Fig. 3** Patterns observed in a 5,500–3,100 Å particle mixture. *a* Shows the pattern of the first plane, and *b* that of the second plane which lies about 4,000 Å above the first plane. Small particles in the first plane are at the centres of triangles of large particles, whereas those in the second plane lie at the midpoints of the lines connecting the blurred images of the larger particles. This is characteristic of an AB<sub>5</sub>-type alloy structure.

Figure 3*a* shows a hexagonally ordered structure which appeared in a 5,500–3,100 Å system, the particle-number ratio of which was 1:2. On moving the microscope focus upwards by 0.8 μm, the next plane of the same arrangement appeared. All the particles, large and small, were directly above those in the first plane; no staggering was found. Then, moving the microscope focus down from this plane very carefully, another new plane was found lying between the above-mentioned two planes (see Fig. 3*b*), but in this new plane the smaller particles were arranged in a different way from those in Fig. 3*a*. The larger particles in Fig. 3*b* are out of focus, indicating that they are not in this plane. This new plane seems to consist solely of 3,100 Å particles, for if there were 5,500 Å particles here, they would have to lie between two infocused large particles whose centres are only 0.8 μm apart, which is impossible. Figure 4 shows the particle arrangement in the two planes. The superstructure must be composed of a combination of these two planes. Brief consideration leads to the formula AB<sub>5</sub> as the composition. This again corresponds to the structure of an intermetallic compound, in this case, CaCu<sub>5</sub> (ref. 10). This resemblance between the superstructure in latex and those of some alloys is very interesting. These latex superstructures may, therefore, be called 'colloid alloys'.

Because the electrolyte concentration is very low in the present systems ( $\sim 10^{-5}$  mol l<sup>-1</sup>) the colloid interaction between the particles must be repulsive, and in general, repulsive forces work to make the whole system uniform, and do not explain the



**Fig. 4** Illustrative drawing of the AB<sub>5</sub>-type structure. The inter-layer spacing is exaggerated for convenience. Open and solid circles represent the 5,500 Å and 3,100 Å particles, respectively.

occurrence of phase separation. Therefore, the mechanism of formation of these structures must be due to some entropy effect<sup>2,3</sup>. This means that in intermetallic compounds of this kind, the stoichiometry is determined by the size ratio of the component atoms; the electrons act only as a cement to hold the atoms together, and this cementing action can be replaced by pressure.

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## Influence of stratospheric cooling from CO<sub>2</sub> on the ozone layer

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**Perturbation of the atmospheric temperature distribution caused by an increased content of CO<sub>2</sub> is a key problem in climate research. The main concern is the increase in temperatures at the Earth's surface due to an increased greenhouse effect. A general cooling of the stratosphere is predicted to take place when CO<sub>2</sub> increases. A direct effect of lower temperatures in the stratosphere is a change in the rates of some of the chemical reactions which control the ozone layer. Anthropogenic release of carbon dioxide may therefore lead to changes in the ozone layer in the future. The present paper shows that estimates of the temperature effect on ozone depend markedly on the level of chlorine in the stratosphere, as the catalytic ozone reduction by nitrogen and chlorine reactions respond differently to changes in temperature.**

Model estimates<sup>1–3</sup> predict an increase in the stratospheric ozone content with decreasing temperatures. This would mean that future decreases in stratospheric ozone due to fluorocarbon releases might have considerably fewer environmental consequences due to an effect opposite to that of CO<sub>2</sub>. In particular Groves *et al.*<sup>3</sup> find the effect from increased CO<sub>2</sub> to be significant compared with future chlorofluoromethane (CFM) effects. To estimate the effect of reduced temperatures on stratospheric ozone, they used a one-dimensional time-dependent model with temperature feedback included. We have used a similar chemical transport model, with prescribed temperature profiles, to repeat their calculations of the temperature effect on ozone. Our calculations give smaller changes in total ozone.

The one-dimensional time dependent model is the same as that used by Hessvedt and Isaksen<sup>4</sup>, with updated rate constants. Groves *et al.*<sup>3</sup> calculate the effect of increased CO<sub>2</sub> on total ozone in a model which does not include chlorine reactions, while our model takes the chlorine chemistry into account. To

compare the models from a technical point of view, we calculated the effect of reduced temperatures corresponding to doubled CO<sub>2</sub> content in the atmosphere (600 p.p.m.), neglecting the chlorine chemistry. We also adjusted the rate constants for the more important reactions (the reactions of HO<sub>2</sub> with NO and O<sub>3</sub>) in accordance with the values used by Groves *et al.*<sup>3</sup>. The resulting increase in total ozone density agrees well with the increase obtained by Groves *et al.*—5.6% compared with 5.5%. Calculated increase in ozone with height in the stratosphere is also found to be in good agreement in the two models. Thus the smaller increase in ozone obtained in our model is probably a result of a more complete ozone chemistry obtained through consideration of chlorine reactions. This point is discussed further below. Groves and Tuck<sup>5</sup> have recently shown that the temperature effect also becomes smaller when chlorine reactions are included in their model.

Future effects of CO<sub>2</sub> on ozone are obtained by two sets of calculations. Chlorine variations are the same in the two sets of calculations, determined by the release of CFMs, while we use two different temperature profiles. In the first set of calculations a standard temperature profile is used. In the second a different profile is used in the stratosphere, corresponding to doubling (600 p.p.m.) of CO<sub>2</sub> in the atmosphere. The difference in total ozone between the two sets of calculations at a certain year should therefore give the effect of the adopted temperature change for a chlorine content corresponding to that year. In which year this will take place, will depend on the future release rates of CO<sub>2</sub>, Freon 11 and Freon 12 (F-11 and F-12). The temperature profile we adopt, corresponding to doubled CO<sub>2</sub> in the atmosphere, is the same as that calculated and used by Groves *et al.*<sup>3</sup>. It gives a temperature reduction of 4°C at 25 km, 6°C at 30 km, 10°C at 40 km, and 13°C at 50 km. These values refer to summer conditions. During the winter reductions are somewhat lower. The numbers are in good agreement with stratospheric cooling obtained by Manabe and Wetherald<sup>6</sup>.

In the present model increase in the stratospheric chlorine content is calculated from a yearly release of F-11 and F-12 up to 1977<sup>7</sup> which is approximately 85–90% of the production the same year. After 1977 constant release rates, equal to the 1977 production are adopted. Table 1 gives the calculated increase in total ozone density (in per cent) which would result from a doubling of the CO<sub>2</sub> in different years. As the change in ozone for each year is calculated with the same amount of chlorine present, the value in Table 1 gives the effect of temperature alone on ozone, at that certain level of chlorine.

The value for year 1955 refers to the chlorine content in the atmosphere before it was affected by release of F-11 and F-12. The source for chlorine before 1955 is assumed to be release of CH<sub>3</sub>Cl and CCl<sub>4</sub>. Both these compounds are assumed to be of natural origin and therefore to have constant release rates. For the years after 1955 Table 1 gives the amount of ClX, which is calculated to be present in the stratosphere above 30 km as a function of time. The temperature effect is seen to become less pronounced the more chlorine there is in the stratosphere. With the amount of chlorine estimated to be in the stratosphere in year 2040, a doubling of the CO<sub>2</sub> in the atmosphere leads to an increase in total ozone of 2.8%, slightly more than half the increase we obtain for a negligible chlorine content.

**Table 1** Calculated increase in total ozone versus time resulting from the temperature changes given by a doubling of the CO<sub>2</sub> content in the atmosphere at various chlorine concentrations

| Year                                | 1955 | 2000 | 2020 | 2040 |
|-------------------------------------|------|------|------|------|
| ClX (parts per 10 <sup>9</sup> )    | 0    | 1.0  | 2.6  | 3.5  |
| ΔO <sub>3</sub> /O <sub>3</sub> (%) | 5.1  | 4.3  | 3.3  | 2.8  |

The ClX (Cl + ClO + HCl + ClONO<sub>2</sub>) mixing ratios are given for heights above 30 km, and are calculated in the model assuming a constant release rate of CFM at the 1977 level. The increases shown are for summer conditions.

**Table 2** The ozone loss at various heights in the stratosphere in % of total loss at each height

| Height (km) | Chemical cycles |           |           |           |
|-------------|-----------------|-----------|-----------|-----------|
|             | Oxygen*         | Hydrogen† | Nitrogen‡ | Chlorine§ |
| 45          | 16.1            | 24.9      | 9.5       | 49.5      |
| 40          | 8.7             | 8.3       | 16.3      | 66.7      |
| 35          | 7.3             | 5.6       | 23.8      | 63.2      |
| 30          | 8.1             | 10.4      | 22.5      | 59.0      |
| 25          | 6.7             | 29.8      | 12.9      | 50.6      |
| 20          | 3.1             | 74.6      | 5.8       | 16.5      |

The loss in each group is obtained from the following reactions:

\* Oxygen: O + O<sub>3</sub> → 2O<sub>2</sub>

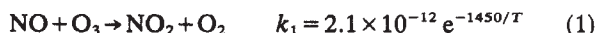
† Hydrogen: HO<sub>2</sub> + O → OH + O<sub>2</sub> and HO<sub>2</sub> + O<sub>3</sub> → OH + 2O<sub>2</sub>

‡ Nitrogen: NO<sub>2</sub> + O → NO + O<sub>2</sub>

§ Chlorine: ClO + O → Cl + O<sub>2</sub>

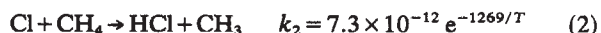
The values refer to the estimated stratosphere chlorine content for the year 2040, and are for the summer season.

We find this marked reduction of the effect of temperature changes, when the chlorine content is increased, to result from the different ways nitrogen and chlorine reactions respond to temperature changes. In the nitrogen cycle the ozone loss is proportional to the concentrations of NO<sub>2</sub>. Reduced temperatures reduce the production of NO<sub>2</sub> from NO:



thereby lowering the equilibrium concentrations of NO<sub>2</sub>. This makes the nitrogen cycle less effective, with the result of increased concentrations of ozone.

In the chlorine cycle where there is chemical equilibrium between Cl, ClO, and HCl, a change in the temperature profile first of all affects the reaction:



This reaction converts active chlorine, Cl and ClO, to HCl which is inactive with respect to ozone destruction. A temperature reduction in the stratosphere will therefore increase the ozone destruction by chlorine reactions. The calculations show that a temperature reduction of approximately 10 K at 40 km reduces the NO<sub>2</sub> densities, and thereby the ozone destruction through the nitrogen cycle by approximately 10%. It increases the ClO densities, and thereby the ozone destruction through the chlorine cycle by approximately 6%. Similar changes in NO<sub>2</sub> and ClO are found at other heights. Other species are found to be little affected by changes in temperature.

When the chlorine chemistry is considered in the model, the importance of the nitrogen cycle for the ozone distribution is markedly reduced. This will particularly be true when high chlorine build up takes place during the next century as we have predicted in Table 1. This is clearly illustrated in Table 2 where the loss (in relative units) due to the different chemical cycles is given. The numbers refer to the year 2040. In the region between 30 and 40 km where the temperature effect from a CO<sub>2</sub> increase has the largest impact on total ozone, the catalytic chlorine reactions are responsible for approximately 60% of the ozone destruction. It is therefore not surprising that the temperature effect on ozone is found to vary considerably with varying chlorine content in the stratosphere. A consequence of this is that comparisons of future effects of CO<sub>2</sub> and CFM also must take into account how long it takes for CO<sub>2</sub> to double its atmospheric concentrations, and how accurate the adopted release rates of CFM are. In these comparisons we have assumed that CO<sub>2</sub> will reach 600 p.p.m. around year 2040. Unfortunately this assumption, and also the assumption of a future release rate of CFM on the 1977 level are both highly uncertain.

In order to compare the effect of future increase in atmospheric CO<sub>2</sub> with the effect of increased CFM levels, estimated reductions of total ozone due to constant future CFM release on the 1977 level are given in Table 3. The calculations show that there will be a marked decrease in ozone well into the next



**Table 3** Calculated changes in total ozone concentrations versus time due to the release of CFMs (F-11 and F-12)

| Year                 | 1978 | 2000 | 2020  | 2040  | $\rightarrow\infty$ |
|----------------------|------|------|-------|-------|---------------------|
| $\Delta O_3/O_3$ (%) | -1.9 | -7.4 | -11.1 | -13.9 | -23.4               |

Release rates up to 1977 are taken from ref. 7. A constant release rate corresponding to the production in 1977 is adopted for the years after. Effects from changes in stratospheric temperatures are neglected. The changes shown are for the summer season.

century. The reduction due to CFM release in year 2040, which is of special interest when comparisons with the  $CO_2$  effect is made, is approximately 14%. Compared with the 2.8% increase in total ozone from a doubling of  $CO_2$  we obtained above, we find the temperature effect due to increased  $CO_2$  to be only an adjustment of the CFM effect. Note, however, that the above values are likely to represent overestimates of the CFM effect as we have not considered any feedback from reduced  $O_3$  in the stratosphere to the temperature profile. However, estimates of this effect<sup>8</sup> indicate that it leads to a small change in the CFM reduction of ozone. The calculations of Groves and Tuck<sup>5</sup> show considerably smaller future reductions in total ozone when the combined effect of  $CO_2$  and CFM is considered, than those obtained in our calculations.

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## Nitrogen fertiliser and stratospheric ozone: latitudinal effects

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Substantial increases in atmospheric nitrous oxide ( $N_2O$ ) resulting from increased use of nitrogen fertilisers might cause large (to 10%; ref. 1) and unacceptable decreases in the stratospheric ozone content<sup>1-3</sup>. Such ozone decreases would be caused by catalytic reaction cycles involving odd-nitrogen ( $NO_x = NO + NO_2$ ) which is formed by  $N_2O$  decomposition in the upper stratosphere<sup>1</sup>. However, Turco *et al.*<sup>4</sup>, using a background chlorine level of 2 p.p.b.v. (parts per billion by volume), show that if the recently measured values of the reactions in Table 1 are used, a 50% increase in  $N_2O$  would lead to a 2.7% increase in the stratospheric column density, although the ozone content above 30 km would be reduced by > 5%; they also estimated (unpublished data) that the change in the ozone column density caused by doubling the  $N_2O$  abundance would be very close to zero (within about 0.1%). Here we extend these calculations of  $N_2O$ /ozone effects to two dimensions, thereby delineating the latitudinal dependence expected for such ozone perturbations; we also discuss the effects of changes in stratospheric chlorine levels ( $Cl_x \equiv Cl + ClO + HCl$ ) on predicted ozone changes.

The two-dimensional model used in this study extends from the surface to 60 km altitude and from latitude 80° N to 80° S with 2.5 km vertical grid and 5° latitudinal grid. About 35 constituents in the oxygen-nitrogen-hydrogen-chlorine-carbon families are included. Transport is simulated by advection and eddy diffusion. Detailed discussions of the model are given elsewhere<sup>7,8</sup>.

We have made model calculations that simulate a doubling of atmospheric  $N_2O$  for two possible levels of stratospheric chlorine content, 1.4 p.p.b.v. and 2.1 p.p.b.v., at 60 km altitude. The corresponding predicted changes in ozone abundances are shown in Fig. 1 as a function of altitude and latitude. The most notable feature of these profiles is the substantial ozone increase predicted for the lower stratosphere (up to 10% below 20 km at high latitudes, and up to 15% below 27 km at low latitudes). The ozone gains are caused principally by the interference by enhanced  $NO_x$  concentration in the  $HO_x$  reaction cycle that consumes ozone; that is, through interference caused by the coupling reaction (see Table 1)



This occurs via two major mechanisms. One is accelerated  $HO_x$  recombination through reactions involving nitric acid

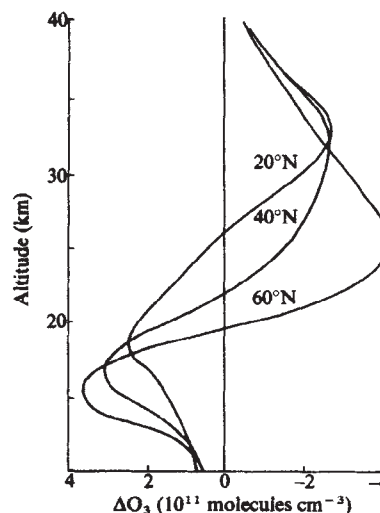


The second suppresses  $HO_x$  catalysis of ozone by direct short circuiting of the reaction



by reaction (1) (see Table 1). Eventually, as  $NO_x$  is increased beyond some level, enough of the OH is taken up by nitric acid and destroyed by the reaction  $OH + HNO_3$  that it decreases as  $NO_x$  is increased. Our computations, in fact, show that OH may be reduced by nearly  $10^6$  molecules  $cm^{-3}$  (by about 25%) for  $N_2O$  doubling, especially at mid-latitudes and high latitudes. Reductions in OH have, of course, consequences for the lifetimes of other stratospheric constituents, including many hydrocarbons and halocarbons.

Figure 2 shows the predicted latitudinal variation of the relative (per cent) ozone column density change for two levels of stratospheric chlorine (1.4 p.p.b.v. and 2.1 p.p.b.v.). The important features to be noted here are the minima that may occur at low latitudes, and the pronounced influence of the ambient stratospheric chlorine level. The low latitude minima would occur partly because of stratospheric transport, which



**Fig. 1** Predicted steady-state change in stratospheric ozone concentrations at three latitudes when the atmospheric  $N_2O$  abundance is doubled. The level of stratospheric chlorine is 2.1 p.p.b.v.

**Table 1** Some key reactions

| Reaction                                                           | Rate coefficient*                                     | Ref. |
|--------------------------------------------------------------------|-------------------------------------------------------|------|
| (1) $\text{NO} + \text{HO}_2 \rightarrow \text{NO}_2 + \text{OH}$  | $8 \times 10^{-12}$                                   | 5    |
| (2) $\text{HO}_2 + \text{O}_3 \rightarrow \text{OH} + 2\text{O}_2$ | $1.4 \times 10^{-14} \exp\left(\frac{580}{-T}\right)$ | 6    |

*T* is the absolute temperature in degrees Kelvin.

moves relatively inert substances (tracers), such as ozone and total odd-nitrogen, from low to high latitudes<sup>9</sup>; as a result,  $\text{NO}_x$  increases at higher latitudes. On the other hand, increases in the background level of chlorine are predicted to cause less ozone destruction due to increase of  $\text{N}_2\text{O}$ , mainly because of the reaction



and to a lesser extent through the formation of chlorine nitrate ( $\text{ClONO}_2$ ). As  $\text{NO}_x$  is increased, this strong  $\text{Cl}_x$ - $\text{NO}_x$  coupling reaction significantly slows the rate of ozone catalysis by ambient chlorine. In other words, the addition of  $\text{NO}_x$  can render the ambient chlorine catalytic cycle less effective through reaction (3) and can, if the chlorine concentration is high enough, result in an ozone increase. Hence the complex interplay among the  $\text{NO}_x$ ,  $\text{HO}_x$ , and  $\text{Cl}_x$  reaction cycles (also discussed in ref. 10) at first leads to a net ozone increase as  $\text{N}_2\text{O}$  burdens are increased, which later 'saturates' and becomes a net ozone decrease. This effect was predicted by Turco *et al.*<sup>4</sup> for aircraft injections of  $\text{NO}_x$  into the stratosphere. The present calculations show that the saturation point is indeed very sensitive to the ambient chlorine level, and is not uniform over the globe. Note also that this prediction is highly sensitive to other model parameters, including the ambient water vapour concentration and the rates of several key reaction processes such as reactions (1) and (2). Qualitative estimates of the rates of odd-oxygen (ozone) consumption for the key catalytic reaction steps are presented in Fig. 1 of ref. 4. The global average ozone changes shown in Fig. 2 are roughly consistent with those obtained with our one-dimensional model<sup>4</sup>. Note that the hemispherical asymmetry in Fig. 2 is a consequence of the asymmetry in eddy transport required for matching observed ozone abundances by model predictions. The small differences between the two models are due mainly to differences in effective vertical transport. That is, for the high

chlorine case, we obtain an annually and globally averaged ozone change of about -0.5%; the corresponding prediction with the one-dimensional model is zero change. The difference is due to a somewhat different mean vertical transport rate between the two models.

The cycle of atmospheric nitrous oxide is not well understood. A major controversy centres on the possibility of a large unidentified tropospheric sink for  $\text{N}_2\text{O}$ . If the magnitude of that sink is as large as has been suggested by Hahn<sup>11</sup>, it is unlikely that the abundance of atmospheric  $\text{N}_2\text{O}$  will double in the next 50 years. Inasmuch as stratospheric levels of chlorine are expected to exceed about 2 p.p.b.v. in future decades, our calculations suggest that global ozone reductions due to large  $\text{N}_2\text{O}$  increases could be quite small, and that they should be most pronounced at high latitudes; at low latitudes, ozone column increases are actually likely to occur. Thus, because the atmospheric lifetime of  $\text{N}_2\text{O}$  is probably not much larger than 10 years, we apparently have the time to carry out an extended and detailed scientific analysis of the global  $\text{N}_2\text{O}$  budget before critical decisions concerning the regulation of nitrogen fertilisers will be necessary.

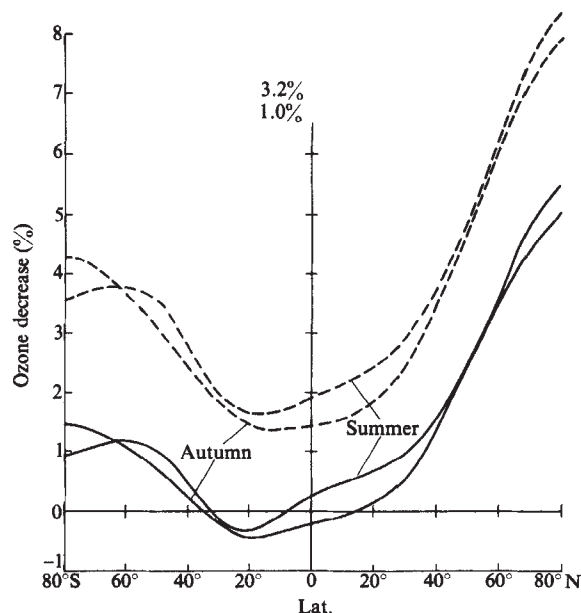
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## Ethylene evolution by thrips-infested cowpea provides a basis for thrips resistance screening with ethephon sprays

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**Fig. 2** Predicted latitude dependence of the change in the ozone column density for the summer and autumn seasons and for two levels of stratospheric chlorine:  $\text{Cl}_x = 1.4$  (dashed line) and 2.1 p.p.b.v. (solid line).

Pests are major constraints to increased cowpea production in tropical subsistence agriculture where cash inputs are small. Breeding for pest resistance is therefore the major component of pest management strategies for crop improvement. Of paramount economic importance among these pests are flower thrips [*Megalurothrips sjostedti* (Tryb.)] which may cause abscission of flower buds, flowers and peduncles, leading to seed yield losses of up to 100% (refs 1, 2). It has been difficult to use available sources of moderate thrips resistance in a breeding programme because of thrips variation in time and space in field screening<sup>3</sup>. This paper reports that ethylene is produced when thrips infest cowpea peduncles. We have used this fact to develop a screening technique using the synthetic growth regulator ethephon (2-chloroethylphosphonic acid), which when sprayed on the plant is translocated to actively-growing tissue, where it breaks down to form ethylene<sup>4,5</sup>. Cultivars susceptible to abscission caused by thrips also show increased abscission with ethephon treatment. The technique may be useful in identifying sources of resistance to other abscission-causing agents.

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**Table 1** Peduncle abscission and flower budset on the main stem of lines with contrasting resistance level to *Megalurothrips*, when sprayed with one of three concentrations of ethephon in two field experiments

| Ethephon (p.p.m.)                 | Peduncle abscission (%) |      |      |        |      |      | Flower budset (%) |      |      |        |      |      |
|-----------------------------------|-------------------------|------|------|--------|------|------|-------------------|------|------|--------|------|------|
|                                   | Expt 1                  |      |      | Expt 2 |      |      | Expt 1            |      |      | Expt 2 |      |      |
|                                   | 0                       | 200  | 400  | 0      | 200  | 400  | 0                 | 200  | 400  | 0      | 200  | 400  |
| Five thrips-susceptible cultivars | 1.2                     | 48.6 | 67.0 | 14.4   | 78.8 | 91.4 | 54.6              | 25.4 | 12.8 | 49.8   | 5.0  | 1.6  |
| Five thrips-resistant cultivars   | 0.4                     | 7.4  | 22.6 | 0      | 7.0  | 35.6 | 71.0              | 62.6 | 30.4 | 76.2   | 50.4 | 15.6 |
| Mean of 30 cultivars              | 1.2                     | 35.7 | 53.3 | 4.0    | 50.2 | 75.0 | 62.9              | 32.9 | 16.5 | 62.9   | 19.4 | 5.7  |
| s.e.m., sprays                    |                         | 6.2  |      |        | 1.9  |      |                   | 6.0  |      |        | 2.1  |      |
| s.e.m., cultivars                 |                         | 6.0  |      |        | 4.8  |      |                   | 5.7  |      |        | 4.3  |      |
| c.v. (%)                          |                         | 49.1 |      |        | 27.0 |      |                   | 37.5 |      |        | 36.4 |      |

Evidence that thrips damage can lead to ethylene production was obtained when pot-grown plants of two cowpea cultivars (*Vigna unguiculata* L. Walp.), raised in insect-free conditions, were transferred to a thrips-infested cowpea field and ethylene evolution of the peduncles was measured. Half the plants were treated with the systemic insecticide monocrotophos 1 day before transfer to the field. The insecticide-treated plants remained free of thrips, while unsprayed plants showed an average of three adult thrips and six nymphs per peduncle 4 days after field transfer. At that time, when flower bud abscission of the unsprayed plants was just beginning, three peduncles were removed from six plants in each treatment and incubated at 23 °C in 10-ml plastic syringes under 0.75 bar suction tension. To absorb CO<sub>2</sub> given off in respiration, 0.2 g solid KOH was placed in a perforated plastic vial in each syringe. After 24 h incubation, a 0.5-ml gas sample from the syringe was injected in a gas chromatograph for determination of the presence of ethylene<sup>7</sup>. Thrips-infested peduncles produced 640 nmol ethylene per g fresh weight per 24 h and the controls produced 152 nmol. The differences are significant at the 1% level. There was no difference between the two cultivars.

Therefore, it seems that thrips infestation leads to ethylene evolution. As no attempt was made to remove the thrips from infested peduncles before incubation, the source of the ethylene still remains to be determined. The most likely source would be ethylene evolved by the outer cell layers of the flower buds and peduncles that had been injured by the thrips<sup>8,9</sup>. Ethylene could also be produced by the plants as a result of interactions with chemicals secreted by the insects, as occurs in cotton<sup>10</sup>.

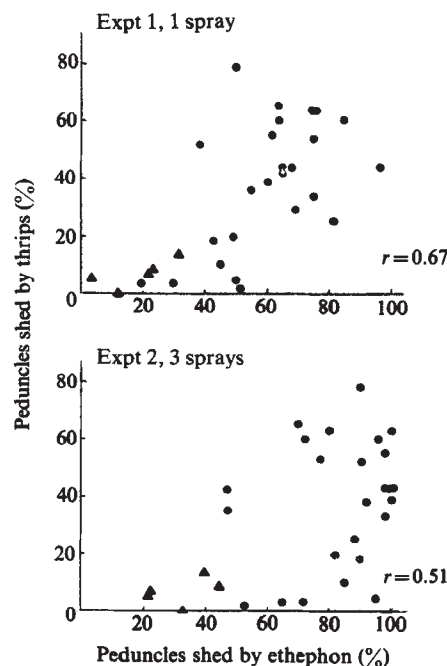
Can applications of the ethylene-producing chemical ethephon simulate thrips damage, and identify cultivars resistant to thrips? To answer these questions two field experiments, comparing flower bud and peduncle abscission of 30 cowpea lines were planted in first and second rains at IITA, Ibadan. Plots consisted of single rows 1 m apart and 3 m long, replicated four times. Both experiments were sprayed weekly with insecticides starting at 2 weeks after sowing. Applications of 0, 200 or 400 p.p.m. ethephon in water containing 0.5% (v/v) of a wetting agent formed the main plots, with cultivars as subplots in the split plot experiments. In experiment 1, a single ethephon spray was applied at about 1 week before anthesis of a particular cultivar. At this stage, the oldest peduncles were about 1 cm long. In experiment 2, all cultivars were sprayed 34, 39 and 47 days after sowing with 0, 200 or 400 p.p.m. ethephon, to ensure that each cultivar received at least one spray during the period of maximum sensitivity to abscission. In both experiments the five lowest peduncles on the main stem of three plants per plot were examined for peduncle or flower bud abscission 2–4 days after the controls started flowering. Only the lowest two buds per peduncle were considered in rating flower bud abscission. The number of plants flowering was counted on each plot at 2–3 day intervals.

The same 30 cultivars were field tested for thrips resistance in four replications in late second rains without insecticide protection (experiment 3). Flower thrips infestation was heavy and

completely prevented flowering of some cultivars. After 50 days, thrips susceptibility was measured by counting the number of abscised peduncles on the first five nodes of the main stem of three plants per plot.

In experiments 1 and 2 ethephon sprays resulted in increased peduncle abscission and reduced budset in all cultivars, with thrips-susceptible cultivars being most severely affected (Table 1). Thrips-resistant cultivars showed less damage than susceptible lines with 200 and 400 p.p.m. ethephon (spray treatment × cultivar interaction was significant at the 1% level). There was no evidence of enhanced fruitset at concentrations of 250–500 p.p.m. as reported for *Phaseolus vulgaris*<sup>11</sup>.

The correlation between per cent peduncle abscission due to ethephon and that due to thrips was 0.75 and 0.62 for experiments 1 and 2, respectively (Fig. 1). In both experiments, five cultivars showed good levels of resistance to ethephon and to thrips, indicating that selection of plants with low main stem peduncle abscission after sprays of 400 p.p.m. ethephon can identify individuals with thrips resistance. Five other cultivars showed low incidence of peduncle abscission due to thrips after one spray of 400 p.p.m. ethephon, but less tolerance to repeated applications of the chemical. Such cultivars may be less resistant to higher thrips populations than occurred in experiment 3.



**Fig. 1** The relationship of peduncle abscission caused by thrips and by 400 p.p.m. ethephon for 30 cowpea lines grown in two field experiments. Points marked by a triangle represent lines that have shown consistent thrips resistance in other experiments.

It seems clear that ethephon sprays can assist in identifying cowpea lines which possess flower thrips resistance. The sprays can be applied uniformly, and as resistance is built up through intercrosses of different resistant parents, the dosage level could be increased to simulate heavier thrips pressure. The thrips resistance of such material would require verification, for in large plantings of a resistant cultivar, thrips levels may build up sufficiently to cause damage.

The use of ethephon as a screening tool for abscission resistance could have important implications for other crop improvement programmes. Various insect pests are known to cause abscission of young reproductive structures in cotton<sup>11-13</sup> and plum<sup>14</sup>, while drought, high temperatures and shade may cause similar damage in edible legumes<sup>7,15,16</sup>. Increases in evolution of ethylene may result from insect damage<sup>11</sup>, shading, drought<sup>17,18</sup> or plant disease<sup>19</sup>.

Thus the abscission response of cultivars to different levels of applied ethephon might identify lines with better tolerance to such pests and stress conditions. Although some authors have pointed out cultivar differences with regard to abscission of reproductive structures<sup>20,21</sup> the systematic screening for cultivars less susceptible to abscission has to our knowledge not been previously reported and seems a promising way of overcoming the problems of abscission-causing insect pests and physical factors.

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## Is prostacyclin a physiologically important circulating anti-platelet agent?

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Prostacyclin (PGI<sub>2</sub>) is a naturally occurring arachidonic acid metabolite which causes relaxation of vascular smooth muscle<sup>1</sup> and is the most potent inhibitor of platelet aggregation known<sup>2</sup>. The anti-aggregatory effects of PGI<sub>2</sub> result from its ability to stimulate platelet adenylate cyclase and elevate platelet cyclic AMP levels<sup>3,4</sup>. The possibility that PGI<sub>2</sub> is a circulating hormone that acts to inhibit platelet aggregation *in vivo* was first suggested by Gryglewski *et al.*<sup>5</sup> and Moncada *et al.*<sup>6</sup>. In their studies using heparinised rabbits and cats, exogenous PGI<sub>2</sub>

caused a decrease in superfused *ex vivo* tendon weight, and this effect was inhibited by infusion of antiserum directed against a stable PGI<sub>2</sub> analogue (5,6-dihydro-PGI<sub>2</sub>). Tendon weight gain occurred in the absence of exogenous PGI<sub>2</sub> and was enhanced by infusion of PGI<sub>2</sub>-binding antibodies, an effect that was more pronounced in arterial than in venous blood. In contrast, Smith and coworkers<sup>7</sup> observed that their PGI<sub>2</sub>-binding antibodies (antiserum directed against 6-keto-PGF<sub>1α</sub>) suppressed the vasodepressor effects of exogenous PGI<sub>2</sub> infused into cats but did not alter blood pressure in the absence of exogenous PGI<sub>2</sub>. They concluded that PGI<sub>2</sub> is not a circulating vasodepressor hormone. The present studies were designed to examine the physiological role, if any, of circulating PGI<sub>2</sub> in the regulation of human platelet function. Here, we report the results of experiments dealing with the effects of PGI<sub>2</sub> and antibodies directed against 5,6-dihydro-PGI<sub>2</sub> on human platelet function and cyclic AMP levels. These studies indicate that, in humans, PGI<sub>2</sub> is not a physiologically important circulating inhibitor of platelet aggregation.

Antibodies which neutralise the effects of PGI<sub>2</sub> were raised in rabbits using the stable PGI<sub>2</sub> analogue 6α, H-5,6-dihydro-PGI<sub>2</sub> (PGI<sub>1</sub>) as the hapten. The preparation of these antibodies and their detailed serological specificity has been reported previously<sup>8</sup>. The γ-globulin fractions of the immune plasma (anti-PGI<sub>1</sub>) and control rabbit plasmas were purified by precipitation with 40% ammonium sulphate. The insoluble proteins were dissolved in 0.14 M NaCl and 0.01 M Tris (pH 7.4) containing 0.1% gelatin and dialysed against this buffer. The final γ-globulin concentration was equivalent to that in the unextracted plasma. Platelet aggregation was quantified by measuring the maximal slope of aggregation after addition of ADP<sup>9</sup>. Platelet cyclic AMP was measured using a commercially available radioimmunoassay kit after acetylation of the unlabelled cyclic AMP<sup>10</sup>. In the cyclic AMP experiments, platelets were separated from plasma by centrifugation through a layer of silicone oil into trichloroacetic acid (TCA). This method, which results in >90% platelet recovery in the TCA layer and separates platelets from plasma in less than 1 min, will be described in detail elsewhere. (M.L.S., in preparation).

Addition of PGI<sub>2</sub> (1 nM) to platelet-rich plasma (PRP) more than doubled the platelet level of cyclic AMP at the first time measured (2.5 min) (Fig. 1). Platelet aggregation induced by

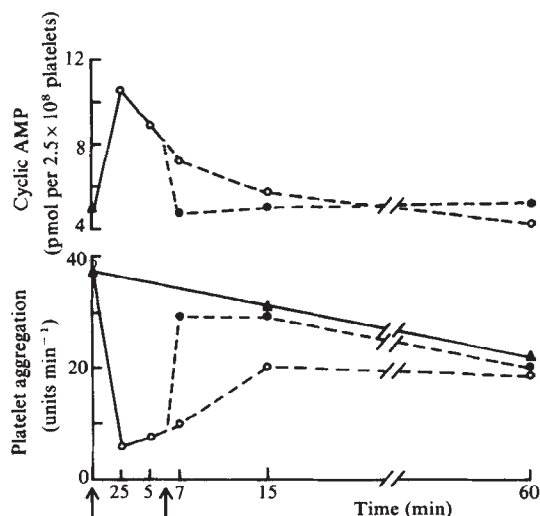


Fig. 1 Typical experiment showing the effect of PGI<sub>2</sub> and antibodies that bind PGI<sub>2</sub> on platelet cyclic AMP levels and ADP-induced platelet aggregation. PGI<sub>2</sub> (1 nM) was added to the PRP at zero time (1st arrow) and 50 μl ml<sup>-1</sup> of either γ-globulin from an immune rabbit (anti-PGI<sub>1</sub>) or γ-globulin from normal plasma was added at 6 min (2nd arrow). Cyclic AMP and platelet aggregation were measured as described in the text. Values represent the mean of duplicate determinations which differed by less than ±10%.

●, Anti-PGI<sub>1</sub>; ○, normal plasma; ▲, no addition.

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**Table 1** Effect of anti-PGI<sub>2</sub> on the increase in rate of ADP-induced aggregation after rapid preparation of platelet rich plasma

| Time after collection (min) | Slope of aggregation (units min <sup>-1</sup> ) |              | Ratio of aggregation slopes at 5 or 10 min versus 3 min |             |
|-----------------------------|-------------------------------------------------|--------------|---------------------------------------------------------|-------------|
|                             | Control                                         | Antibody     | Control                                                 | Antibody    |
| Arterial                    |                                                 |              |                                                         |             |
| 3                           | 20.1 ± 11.0                                     | 18.5 ± 11.4  | —                                                       | —           |
| 5                           | 24.7 ± 8.3†                                     | 23.6 ± 10.8* | 1.39 ± 0.37                                             | 1.42 ± 0.44 |
| 10                          | 27.6 ± 11.1†                                    | 25.4 ± 10.4† | 1.51 ± 0.36                                             | 1.59 ± 0.55 |
| Venous                      |                                                 |              |                                                         |             |
| 3                           | 23.4 ± 10.8                                     | 26.1 ± 9.1   | —                                                       | —           |
| 5                           | 28.4 ± 11.2†                                    | 31.6 ± 11.3* | 1.25 ± 0.20                                             | 1.22 ± 0.19 |
| 10                          | 32.9 ± 9.0†                                     | 32.6 ± 10.4† | 1.57 ± 0.66                                             | 1.29 ± 0.16 |

The PRP was separated from whole blood by high-speed centrifugation. PRP preparation was completed within 2.5 min of blood collection, and 50 µl ml<sup>-1</sup> PRP of either immune or control γ-globulin was added immediately thereafter. This amount of immune γ-globulin had previously been found to reverse the anti-aggregatory effects of 3 nM exogenous PGI<sub>2</sub>. Aggregation, induced by 2 µM ADP, was measured 30 s, 2.5 min and 7.5 min later (that is, 3, 5, and 10 min after blood collection). Results represent mean ± s.d. of duplicate measurements from either 7 (arterial) or 11 (venous) separate experiments using different donors, none of whom had ingested aspirin-containing drugs during the 2-week period before testing. None of the blood samples contained heparin. The slopes of aggregation at 5 min and 10 min with or without antibodies were compared with the comparable values at 3 min. The statistical significance of differences was evaluated using the *t*-test for paired data. Symbols indicate \**P* < 0.05 or †*P* < 0.01 in this comparison. The 5- and 10-min slopes of aggregation were significantly greater than that at 3 min, but there was no difference between controls and samples containing antibodies in this regard.

ADP (2 µM) was almost totally inhibited at this time. Over the next hour, platelet cyclic AMP levels gradually fell to the original level, while the rate of ADP-induced aggregation rose to the control level. Addition of anti-PGI<sub>1</sub> antibodies 6 min after PGI<sub>2</sub> accelerated the fall in cyclic AMP level and the rise in the rate of ADP-induced aggregation, both of which approximated the initial values within 1 min of addition of the antibody. In contrast, addition of the γ-globulin fraction of plasma from a non-immune rabbit had no effect. Addition to control PRP of immune γ-globulin alone, or of PGI<sub>2</sub> previously incubated with these antibodies, did not alter platelet cyclic AMP levels or ADP-induced aggregation. Thus, the anti-PGI<sub>1</sub> antibodies rapidly reverse the anti-aggregatory effects of exogenous PGI<sub>2</sub>.

To investigate whether the sensitivity of platelets to PGI<sub>2</sub> differed in PRP and in whole blood, concentration-response curves for inhibition of ADP-induced aggregation were obtained by addition of exogenous PGI<sub>2</sub> to PRP directly and to whole blood before rapid preparation of PRP. The curves in PRP and in whole blood were virtually identical, and the threshold concentration for inhibition of aggregation was 0.1 nM PGI<sub>2</sub> in both cases. Addition of anti-PGI<sub>2</sub> antibodies to whole blood 15 s after the addition of exogenous PGI<sub>2</sub> (0.1–1 nM) negated the inhibitory effects on platelet aggregation.

If PGI<sub>2</sub> is a physiologically important circulating inhibitor of platelet aggregation, reduction in PGI<sub>2</sub> levels should enhance aggregation. PGI<sub>2</sub> is normally degraded in the circulation with a half-time of 3–5 min (ref. 11), although in citrated plasma the half-time is prolonged to 25–30 min (D.E.M. and E.W.S., unpublished). Previous reports have demonstrated that platelet reactivity *in vitro* increases with time after removal of blood from the vascular system<sup>12,13</sup>. This phenomenon might be explained by time-dependent degradation of PGI<sub>2</sub>. We have also found that the rate of ADP-induced aggregation increases with time after rapid preparation of PRP followed by incubation at 37 °C (Table 1). However, immediate addition of anti-PGI<sub>1</sub> antibodies to PRP prepared within 3 min of venous or arterial puncture did not enhance the rate of ADP-induced aggregation at that time or alter the increase in aggregation over the next

10 min. The PGI<sub>2</sub>-binding antibodies failed to enhance aggregation in either arterial or venous blood (Table 1).

The present studies demonstrate that: (1) the plasma or blood concentration of PGI<sub>2</sub> required for inhibition of ADP-induced aggregation is ≥ 0.1 nM; (2) inactivation of PGI<sub>2</sub> by addition of PGI<sub>2</sub>-binding antibodies to PRP or to whole blood rapidly (<1 min) reverses the effects of exogenous PGI<sub>2</sub> on platelet aggregation and platelet cyclic AMP; (3) addition of PGI<sub>2</sub>-binding antibodies to PRP shortly (<3 min) after either arterial or venous blood collection does not alter platelet aggregation.

If PGI<sub>2</sub> were a physiologically important circulating inhibitor of platelet aggregation in humans, addition of antibodies that neutralise PGI<sub>2</sub> should have enhanced aggregation in these conditions. We conclude that the circulating concentration of PGI<sub>2</sub> in humans is below that required for inhibition of aggregation (<0.1 nM) and that PGI<sub>2</sub> is not a circulating anti-platelet agent in normal conditions in humans. The time-dependent increase in platelet responsiveness after blood collection is therefore not due to degradation of circulating PGI<sub>2</sub>.

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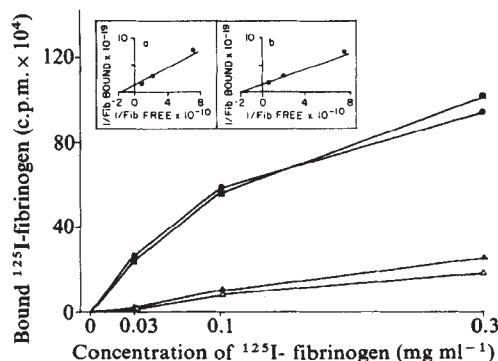
## Prostacyclin inhibits mobilisation of fibrinogen-binding sites on human ADP- and thrombin-treated platelets

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Prostacyclin (prostaglandin I<sub>2</sub>, PGI<sub>2</sub>), produced by the blood vessel wall is the most potent known inhibitor of platelet aggregation induced by such stimuli as ADP and thrombin<sup>1</sup>. It binds to a specific platelet receptor<sup>2</sup> and activates adenylate cyclase, raising the cyclic AMP level in platelets<sup>3,4</sup>. This property can be important because platelets participate in several significant interactions. For example, the interaction with fibrinogen or fibrin contributes to the formation of the haemostatic plug<sup>5</sup>. Intact plasma fibrinogen is required for the aggregation of platelets induced by ADP<sup>6</sup>, and endogenous platelet fibrinogen influences thrombin-induced aggregation<sup>7</sup>. We have therefore studied the effect of prostacyclin on the interaction of fibrinogen with human platelets. We now report that prostacyclin inhibits the mobilisation of specific binding sites ('receptors') for fibrinogen on human platelets and that this effect parallels the inhibition of ADP- or thrombin-induced aggregation. The inhibitory effect of prostacyclin may limit the extent of platelet-fibrinogen interaction *in vivo* and in extracorporeal circulation.

We isolated platelets from human blood and separated them from plasma proteins by stepwise albumin gradient centrifugation and Sepharose 2B gel filtration as before<sup>8</sup>. All experiments were performed with platelets suspended in HEPES buffer, pH 7.35, containing 0.1% dextrose and 0.35% albumin. Aggregation was measured photometrically in a Payton (Buffalo, New York) dual channel aggregometer according to Born's method<sup>9</sup>. Human plasma fibrinogen (Kabi) was prepared and labelled with  $^{125}\text{I}$  as before<sup>10</sup>. This preparation was 95% clottable with thrombin. Solutions of  $\text{PGI}_2$ , 6-keto  $\text{PGF}_{1\alpha}$  and  $\text{PGD}_2$  were prepared in cold Tris-HCl buffer, pH 9.37, immediately before use.



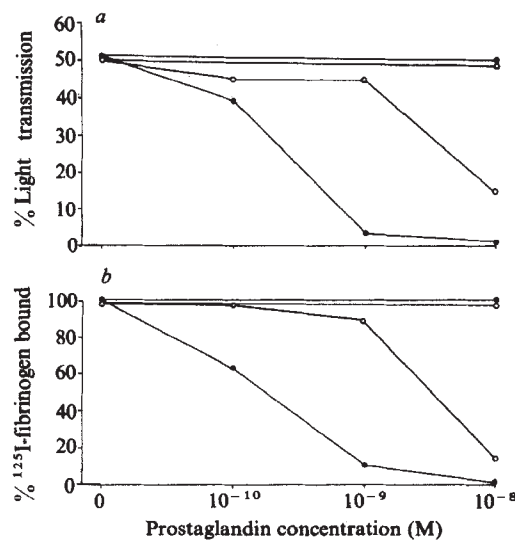
**Fig. 1** Binding of  $^{125}\text{I}$ -labelled human fibrinogen to platelets exposed to: ■, 4.5  $\mu\text{M}$  ADP; ●, thrombin (0.05  $\text{U ml}^{-1}$ ) followed by hirudin (0.1  $\text{U ml}^{-1}$ ); ▲, buffer; ▽, thrombin or ADP and a 20-fold excess of unlabelled fibrinogen. Each incubation included  $1 \times 10^8$  platelets and the specified amounts of fibrinogen ( $4.3 \times 10^4$  c.p.m.  $\mu\text{g}^{-1}$ ) in 0.5 ml of HEPES buffer, pH 7.35, containing 0.1% dextrose and 0.35% albumin. The insets contain double reciprocal plots of binding data for platelets stimulated by ADP and thrombin, respectively. Specific binding was calculated after subtracting values of 'nonspecific' binding in the presence of an excess of unlabelled fibrinogen.

Binding of fibrinogen to platelets was achieved in 0.5-ml reaction mixtures containing  $1 \times 10^8$  platelets with 50  $\mu\text{l}$  of ADP or thrombin preceded or followed by 50  $\mu\text{l}$  of  $^{125}\text{I}$ -fibrinogen. To examine the effect of prostaglandins, platelet suspensions were pre-incubated for 3 min with 50  $\mu\text{l}$  of prostaglandin or buffer before the addition of the other agents. Incubation was at room temperature (22  $^{\circ}\text{C}$ ) in polypropylene tubes (12  $\times$  75) without stirring. After 15 min the mixture was centrifuged through a 9:1 (v/v) mixture of dibutylphthalate and Apiezon oil C (ref. 11). The supernatant was sampled for free radioactivity, and bound radioactivity was measured in the platelet pellet cut off together with the tip of the tube after rapid freezing of its contents in a mixture of dry ice and acetone.

Before examining the effect of prostacyclin on the interaction of  $^{125}\text{I}$ -fibrinogen with human platelets, it was necessary to establish the basic characteristics of binding of  $^{125}\text{I}$ -fibrinogen to human platelets. Intact, resting platelets bound very little  $^{125}\text{I}$ -fibrinogen (Fig. 1). After stimulation with 4.5  $\mu\text{M}$  ADP, there was a four- to fivefold increase in the amount of  $^{125}\text{I}$ -fibrinogen bound. Binding to platelets stimulated with ADP approached saturation at 300  $\mu\text{g}$  of  $^{125}\text{I}$ -fibrinogen, and equilibrium binding was achieved within 5 min. Approximately 70% of the labelled fibrinogen could be displaced by a 20-fold excess of unlabelled fibrinogen, suggesting that binding was specific. Moreover, adenosine, AMP and ATP in equimolar concentrations did not increase the binding of  $^{125}\text{I}$ -fibrinogen to platelets (not shown). The amount of  $^{125}\text{I}$ -fibrinogen bound was calculated from a double reciprocal plot<sup>12</sup> of the binding data obtained after subtracting values for nonspecific binding (Fig. 1, left-hand inset). If we assume uniform labelling of fibrinogen and a molecular weight of 340,000 (ref. 13), we can estimate that

50,000 molecules of  $^{125}\text{I}$ -fibrinogen per platelet were bound to ADP-stimulated platelets with the apparent dissociation constant of  $1.5 \times 10^{-7}$  M.

Thrombin in trace concentrations also stimulates human platelets<sup>14</sup>. To gain further insight into the mobilisation of fibrinogen-binding sites on human platelets we compared the effect of trace amounts of thrombin with that of ADP. In preliminary experiments we determined that a low concentration of thrombin (0.05  $\text{U ml}^{-1}$ ), is blocked by hirudin (0.1  $\text{U ml}^{-1}$ ) in terms of thrombin's action on human fibrinogen. Thus, after exposure of platelets to thrombin, its enzymatic activity toward fibrinogen could be neutralised. After stimulation with thrombin (0.05  $\text{U ml}^{-1}$ ) and then inactivation with hirudin, we observed specific binding of  $^{125}\text{I}$ -fibrinogen with remarkably similar characteristics to those seen after stimulation with ADP (Fig. 1). We estimate that 44,000 molecules of fibrinogen bound with the apparent dissociation constant of  $1.8 \times 10^{-7}$  M (Fig. 1, right-hand inset). Thus traces of thrombin can also induce mobilisation of platelet-binding sites for plasma fibrinogen, a process shown to occur after stimulation of human platelets with ADP<sup>15</sup>. Because thrombin is almost instantly detectable when blood vessels are wounded<sup>16</sup>, our observation that traces of thrombin mobilise fibrinogen receptors in human platelets is important in the platelet-fibrinogen interaction.



**Fig. 2** Concentration-dependent effect of prostacyclin (lower two lines) and 6-keto  $\text{PGF}_{1\alpha}$  (upper two lines) on binding of  $^{125}\text{I}$ -labelled fibrinogen (b) and platelet aggregation (a) induced by ADP (●) and by thrombin (○). For platelet aggregation with ADP, fibrinogen (1.8  $\text{mg ml}^{-1}$ ) was added.

We next examined the possible regulatory role of prostacyclin in this interaction. Prostacyclin (1 nM) inhibited binding of  $^{125}\text{I}$ -fibrinogen to human platelets stimulated with ADP, an effect that was paralleled by inhibition of ADP-induced platelet aggregation (Fig. 2). The dose-response curves for both inhibition of binding of  $^{125}\text{I}$ -fibrinogen to platelets and inhibition of platelet aggregation were similar. Prostacyclin (10 nM) inhibited more than 80% of the binding of  $^{125}\text{I}$ -fibrinogen to human platelets stimulated with thrombin (0.05  $\text{U ml}^{-1}$ ) and also inhibited platelet aggregation induced by the same concentration of thrombin. A 3-min pre-incubation was chosen in consideration of the relatively short lifetime of prostacyclin. However, the inhibitory effect on fibrinogen-binding lasted much longer, up to 60 min after the addition of prostacyclin to platelets.

Our experiments demonstrate that prostacyclin inhibited both ADP- and thrombin-induced mobilisation of binding sites for fibrinogen, which was paralleled by the inhibition of platelet



aggregation and suggests a close association between mobilisation of fibrinogen-binding sites and platelet function. Prostacyclin raises the intraplatelet level of cyclic AMP<sup>3,4</sup>, which can also be attained by PGD<sub>2</sub> (ref. 17). Indeed, PGD<sub>2</sub> (10 nM) also inhibited mobilisation of binding sites for fibrinogen in human platelets (results not shown). These findings suggest that mobilisation and availability of binding sites for fibrinogen are controlled by the cyclic AMP level. A decrease in cyclic AMP, resulting from ADP and thrombin action<sup>18</sup>, promotes mobilisation of fibrinogen-binding sites, important for the subsequent formation of a platelet fibrin plug. An increase in cyclic AMP in response to prostacyclin prevents unmasking of fibrinogen-binding sites.

Although the nature of binding sites for fibrinogen on human platelets is unknown, their number after mobilisation by ADP and thrombin (50,000 and 44,000) is within the range of the 40,000 surface fibrinogen molecules per platelet determined from saturation binding of antifibrinogen Fab fragments<sup>7</sup>. The values established in our experiments are close to the reported number of binding sites induced by ADP<sup>19</sup>. We observed that sites mobilised by ADP and by thrombin are specific, saturable and of high affinity. The process responsible for mobilisation of binding sites of such affinity for fibrinogen can be related to changes in platelets induced by ADP and by a low concentration of thrombin. The changes include transformation from discoid to spherical, protrusion of several pseudopods and distension of the lumen of surface-connecting canaliculi<sup>20</sup>. These changes are accompanied by a decrease in adenylate cyclase activity and by the increase and redistribution of binding sites for lentil lectin demonstrated by Feagler *et al.*<sup>14</sup>. Increased and transient association of fibrinogen with human and rabbit platelets undergoing ADP-induced shape change was observed by Mustard *et al.*<sup>21</sup>.

Note that the platelet-fibrin plug formed at the site of a vascular wound does not expand beyond its immediate zone or occlude the lumen of the injured vessel<sup>5</sup>. The inhibitory effect of prostacyclin produced by the vessel wall on mobilisation of fibrinogen-binding sites on human platelets may limit the size of the platelet-fibrin plug *in vivo*. Furthermore, this inhibitory action of prostacyclin can explain its protective effect on platelet consumption in extracorporeal circulation<sup>22</sup> because the interaction of platelets with foreign surfaces requires the presence of fibrinogen<sup>23</sup>.

Bovine thrombin, hirudin, adenosine, AMP, ADP and ATP were purchased from Sigma. Prostacyclin 6-keto PGF<sub>1α</sub> and PGD<sub>2</sub> were obtained from Drs E. Nishizawa and J. Pike, Upjohn Co. This work was supported by the USPHS grants HL-24119 and HL-08339 and by the Medical Research Service of the Veterans Administration.

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## A Con A-stimulated T-cell hybridoma releases factors affecting haematopoietic colony-forming cells and B-cell antibody responses

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The major role of the thymus-dependent class of lymphocytes (T cells) seems to be regulatory. With the exception of cytotoxic T cells, T-cell subsets act by modulating the function of other T and B lymphocytes<sup>1,2</sup> and macrophages<sup>3</sup>, and also the progenitors of erythrocytes, monocytes and myeloid cells<sup>4–7</sup>. Most of these regulatory effects are mediated by humoral factors, but despite convincing evidence that T cells are essential for the production of such factors, in many instances it has been difficult to demonstrate conclusively that the T cells actually synthesise the factors<sup>7</sup>. Factors stimulating the formation of myeloid colonies in agar can be produced by a T-cell lymphoma, EL4 (ref. 8), and by a hybridoma formed by the fusion of a T-cell lymphoma with pokeweed mitogen-stimulated spleen cells, although in this case it was not possible to demonstrate directly that the non-tumour parent had been a T cell<sup>9</sup>. Here we report that a cloned T-cell hybridoma can be induced to release factor(s) stimulating both the growth of haematopoietic colony-forming cells and the B-cell response to antigen. Incubation of the hybridoma with the T-cell mitogen concanavalin A (Con A), was required for the production of these activities, which were detectable 24 h after addition of the Con A. These activities were not produced when the parental tumour (BW5147) was incubated with Con A.

The hybridoma was formed as described elsewhere<sup>10</sup> by fusing a hypoxanthine-aminopterin-thymidine-sensitive variant of a T-cell lymphoma (BW5147) with blast cells isolated from the lymph nodes of mice immunised with keyhole limpet haemocyanin (KLH)<sup>11</sup>. Hybridomas resulting from the fusion were initially cloned by limiting dilution and the Con A-reactive clone,

**Table 1** The colony-stimulating activity of supernatants of hybridoma cells stimulated with Con A

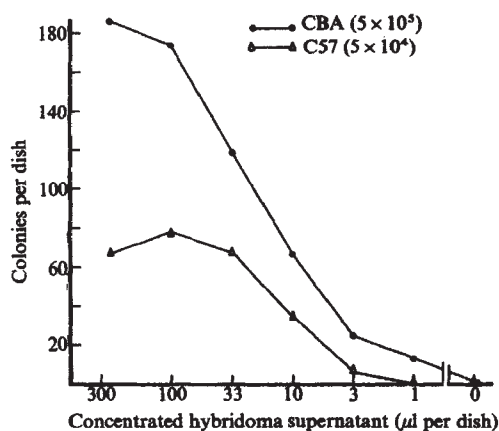
| Supernatant from cultures containing:               | Colonies per dish |
|-----------------------------------------------------|-------------------|
| T-cell hybridoma cells                              | 0, 0              |
| T-cell hybridoma + Con A (0.5 µg ml <sup>-1</sup> ) | 83, 90            |
| T-cell hybridoma + Con A (5 µg ml <sup>-1</sup> )   | 160, 126          |
| TKA                                                 | 0, 0              |
| TKA + Con A (0.5 µg ml <sup>-1</sup> )              | 0, 0              |
| TKA + Con A (5 µg ml <sup>-1</sup> )                | 0, 0              |
| Spleen cells + Con A (5 µg ml <sup>-1</sup> )       | 81, 70            |

Hybridoma cells ( $3 \times 10^5$ ) were incubated for 24 h in 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2-mercaptoethanol ( $5 \times 10^{-5}$  M), L-asparagine ( $3 \times 10^{-4}$  M), glutamine ( $2.8 \times 10^{-3}$  M), 10% fetal calf serum (FCS) and Con A (Pharmacia) at the indicated concentrations. Supernatants were prepared by centrifugation and 0.1-ml aliquots placed in 35-mm plastic Petri dishes to which were added 1-ml aliquots of 0.3% Bacto-agar (Difco) in the medium described above minus 2-mercaptoethanol; each ml contained  $5 \times 10^5$  viable nucleated cells from the femoral shafts of CBA/H/Wehi mice. Also included were control dishes containing 0.1 ml of the supernatants of cultures in which the T-cell lymphoma TKA was incubated with Con A, and control dishes containing 0.1 ml of the supernatant of 48-h cultures of  $50 \times 10^6$  C57BL/6 spleen cells in 10 ml of medium with Con A ( $5 \mu\text{g ml}^{-1}$ ). Colonies were counted with a dissecting microscope on day 7. Morphology of colony cells was determined by plucking individual colonies and then either drying and staining them with aceto-orcein, or smearing and staining them with Giemsa stain.

designated 1.23, was then recloned in agar. It expressed both the Thy 1.1 antigen of the AKR-derived BW5147 tumour and the Thy 1.2 antigen of the CBA lymph-node donor, and possessed 79 chromosomes.

The hybridoma 1.23 did not bind KLH, as determined with fluorescein-conjugated KLH on the fluorescence-activated cell sorter (B.A., F. L. Battye and J. F. A. P. Miller, unpublished observations). Furthermore, the addition of KLH, either in soluble form or presented on peritoneal exudate cells, did not result in the release of regulatory factors. Thus, we have no evidence that the hybridoma 1.23 has receptors for, or is sensitive to, KLH.

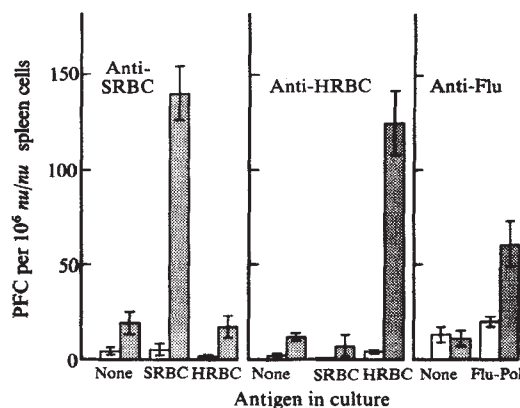
We were aware of the reported instability of T-cell hybridomas. Therefore, we have been careful to store multiple aliquots of both the original clone and subsequently recloned sublines in liquid nitrogen, always recloning in agar after thawing. Experience has shown that the chromosome number is not stable and we have observed chromosome loss in continuously passaged sublines, in some cases without loss of the ability to release regulatory activities. We are exploring the possibility that chromosomal loss may result in functional variants that will aid in the investigation of the number of molecules responsible for the observed biological activities and the mechanism of their release.



**Fig. 1** The colony-forming response of bone marrow from CBA and C57BL/6 mice to supernatants of cultures of Con A-stimulated hybridoma cells. Agar cultures were carried out as described above and contained either  $5 \times 10^5$  viable nucleated CBA bone marrow cells or  $5 \times 10^4$  C57BL/6 bone marrow cells. The hybridoma supernatant was prepared from cells cloned twice in agar and grown in DMEM containing 10% FCS and  $5 \times 10^{-5}$  M 2-mercaptoethanol, by incubating  $50 \times 10^6$  cells in 50 ml of medium without FCS, together with  $5 \mu\text{g ml}^{-1}$  of Con A (Pharmacia). After 24 h the cell-free supernatants were collected by centrifugation and concentrated ninefold in an Amicon stirred cell with a 10,000 molecular weight cutoff membrane (Amicon YM10); they were sterilised by filtration through a 0.45- $\mu\text{m}$  filter (Millipore).

Table 1 shows an experiment demonstrating Con A-dependent secretion by hybridoma 1.23 of factor(s) stimulating the growth of colonies in semi-solid cultures of normal CBA bone marrow cells. Differences were noted in the effects of supernatants of stimulated 1.23 cells on bone marrow cells from two strains of mice. Figure 1 demonstrates the effects of various amounts of concentrated supernatant from a culture of Con A-stimulated hybridoma cells, on the numbers of colonies in cultures of bone marrow cells from two mouse strains, CBA (plated at  $5 \times 10^5$  per dish) and C57BL/6 (plated at  $5 \times 10^4$  per dish). In both cases, colony numbers varied linearly with the

number of cells cultured (data not shown). As shown in Fig. 1, in these culture conditions, the frequency of colony-forming cells was higher (by a factor of 4) in C57BL/6 bone marrow than in CBA bone marrow. Also, C57BL/6 colonies contained either macrophages or granulocytes or both, whereas up to 30% of the CBA colonies contained large multinucleate cells resembling megakaryocytes, together with blast cells and cells with annular or lobed nuclei, the remaining colonies consisting of blast cells and granulocytes or mixtures of granulocytes and macrophages.



**Fig. 2** The effect of supernatants of Con A-stimulated hybridoma cells on the B-cell antibody response to three antigens. Microwell cultures of nu/nu spleen cells containing no antigen, sheep red blood cells (SRBC) ( $10^6$  per well), horse red blood cells (HRBC) ( $10^6$  per well) or fluorescein-conjugated polymerised flagellin (Flu-Pol) (20 ng per well)<sup>16</sup>, were cultured either with (hatched columns) or without (open columns) hybridoma supernatant (20%) for 4 days. Cultures incubated with erythrocyte antigens were divided in half and assayed against both SRBC and HRBC for plaque-forming cells (PFC). Cultures containing Flu-Pol were divided in half and assayed against fluorescein-bearing SRBC<sup>16</sup>, and SRBC, the difference in PFC numbers being shown as Flu-specific PFC. Results are expressed as the mean  $\pm$  s.e.m. of eight replicate cultures. The direct addition of Con A to the cultures, either with or without antigen, had no effect (data not shown). The cultures, in Linbro flat-bottomed trays (76-003-05), contained  $10^6$  viable nucleated BALB/c nu/nu spleen cells in DMEM (0.25 ml) supplemented with 5% FCS,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10 mM HEPES,  $2.8 \times 10^{-3}$  M glutamine and  $3 \times 10^{-4}$  M asparagine. The hybridoma supernatant was prepared and concentrated as described in Fig. 1 legend from cells cloned twice in agar.

Similar results were obtained using medium conditioned by Con A-stimulated spleen cells. Furthermore, there are other indications of differences between various haematopoietic progenitor cells in these two strains. Thus, the granulocyte-macrophage colony-forming cells in C57BL/6 bone marrow are twice as frequent and are much more sensitive to the colony-stimulating factor elaborated by endotoxin-stimulated mouse lung than are those from CBA mice<sup>12</sup>. Furthermore, in cultures stimulated by pokeweed mitogen-stimulated spleen cells, the frequency of mixed or erythroid colonies in fetal liver<sup>13</sup> or adult bone marrow (G. R. Johnson, in preparation) is markedly higher in the CBA than the C57BL/6 strain. Future study should determine both the nature of the colony-forming cells stimulated by the hybridoma supernatants and whether separate molecules are responsible for the growth of the different colonies.

Figure 2 shows the results of an experiment demonstrating that the hybridoma 1.23 supernatant affected B lymphocytes, allowing cultures of spleen cells from congenitally athymic (nu/nu) mice to develop an antibody response to the thymus-dependent antigen, sheep erythrocytes. Both the hybridoma supernatant and the antigen were required for a response. This effect of the supernatant was similar to that achieved with



medium conditioned by Con A-stimulated spleen cells, and seems to be functionally equivalent to the T-cell replacing factor (TRF) described by Schimpl and Wecker<sup>14</sup>. This effect, although requiring the presence of antigen, was non-antigen specific. Thus, the response to a second heterologous erythrocyte, horse erythrocyte, was also enhanced (Fig. 2). The results show that cross-reaction between these antigens was not detected. Furthermore, the response to a third, quite unrelated antigen, fluorescein-conjugated polymerised flagellin (Flu-Pol) was also enhanced. The enhancement of the thymus-independent response to this antigen is consistent with the notion that the hybridoma supernatant is acting subsequent to B-cell activation, amplifying the antibody response in a non-antigen specific way<sup>14,15</sup>.

These observations are interesting from several points of view. First, the availability of a clonal cell line offers great advantages for the reproducible, large-scale preparation of material for biochemical analysis. Preliminary experiments suggest that the hybridoma supernatants also support the *in vitro* proliferation of T lymphocytes and pluripotential stem cells, indicating that several different factors could be involved. It will be important to determine how many molecular species are involved in these activities and, if there are more than one, what relationship there may be between them. In this connection, it is of interest that analyses of medium conditioned by Con A-stimulated spleen cells, the biological activity of which seems to parallel in all respects that of the hybridoma supernatants, show that the B-cell effect and the colony-stimulating activities are separable biochemically, indicating the presence of at least two types of regulatory molecule (I.C.-L. and J.W.S., unpublished data).

Second, the observation that a cloned cell line with definite T-cell markers produces regulatory factors when stimulated with Con A in the same manner as do spleen cell suspensions, indicates that T cells themselves can produce these factors. Third, the demonstration that supernatants derived by stimulation of a cloned cell line enhance antibody responses to several antigens (Fig. 2), is against the notion that the 'T-cell replacing' activity produced by Con A-stimulated spleen is not really non-antigen specific, but is due to the release of a mosaic of antigen-specific T-cell factors on polyclonal stimulation of T cells.

Finally, functional T-cell hybridomas have proven more elusive than their B-cell counterparts. Studies of the conditions required for activation of this hybridoma by Con A may aid in defining conditions for the optimal induction and function of T-cell hybridomas. Furthermore, assays for the release of soluble factors such as those described here may prove useful as screening procedures for the detection of stimulation of T-cell hybridomas by specific antigen.

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## Development of specific suppressor cells in hypoinsulinaemic mice

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Mice and rats injected with alloxan or streptozotocin develop permanent diabetes, characterised by deficient insulin production. It has been demonstrated that hypoinsulinaemia in mice leads to significant loss of lymphatic tissue<sup>1,2</sup>, and these diabetic animals cannot develop contact sensitivity<sup>1</sup> or efficient graft rejection<sup>3,4</sup>. Administration of insulin partially restored these responses<sup>1,4</sup> and also caused an increase in the weight of the thymus and spleen<sup>1</sup>. Similar suppression of T cell-dependent phenomena has been observed in surgically pancreatectomised rats<sup>5</sup>. Lymphocytes of these hypoinsulinaemic animals show significantly decreased *in vitro* responses to plant lectins<sup>2,6,7</sup> and generate only low levels of cytotoxic effector cells<sup>2,7</sup>. We previously showed that cells of normoglycaemic oxazolone-sensitised mice cannot transfer significant contact sensitivity reactions into diabetic recipients<sup>1</sup> indicating that the milieu of hyperglycaemic insulin deficient animals cannot support all the activity of immune T cells. By mixing immunised T cells from control and diabetic mice and transferring the mixtures into normal recipients we now show that the non-supportive milieu in diabetic animals may be due to active suppression rather than to atrepsis.

CBA and DBA/2 inbred male mice were rendered diabetic by intravenous (i.v.) injection of alloxan (75 mg per kg). Only mice with blood glucose levels exceeding 400 mg per 100 ml and showing no signs of diabetic disease were included in the experiments<sup>1</sup>. Normoglycaemic mice were sensitised by application of 0.1 ml of 7% picryl chloride (PCL) or 3% oxazolone (OX) to the skin of the clipped abdomen<sup>8</sup>. Diabetic mice were sensitised 4 days after alloxan administration with lower doses of PCL (3%) and OX (1%) than normal mice because higher doses tend to be toxic in these mice. Normal mice respond similarly to both antigen doses<sup>8,9</sup>. Four days later single cell suspensions were prepared from the spleens and peripheral lymph nodes of the diabetic and control animals. In some experiments cells of diabetic CBA mice were treated with anti-Thy1 serum and complement<sup>10</sup> or T cells were isolated with the help of nylon wool columns<sup>11</sup>. Cells from sensitised normoglycaemic animals ( $4 \times 10^7$ ) were injected i.v. into normal recipients. Some recipients also received  $2 \times 10^7$  cells from diabetic mice which had or had not been previously immunised. Immediately after transfer the mice were challenged on the ear with 1% PCL or OX in olive oil and the increment of ear thickness measured after 24 h<sup>8</sup>.

The results of such an experiment (Table 1) shows that nylon non-adherent Thy 1 positive cells from immunised diabetic mice abrogate in an antigen-specific way the ability of normal immune T cells to transfer contact sensitivity. Thus, a lack of insulin changes an otherwise immunogenic sensitising procedure into one which preferentially activates suppressor T cells.

The induction of specific suppressor cells by antigen in hypoinsulinaemic environment could be explained in a number of ways. One possibility is that distinct subpopulations of T lymphocytes show selective susceptibility to high glucose levels (or high osmotic pressure). This interpretation, although not disproved, seems unlikely since T helper cells function quite well *in vitro* at glucose concentrations much higher than those present in diabetic animals<sup>12</sup>. Another possibility, and the one we favour, is that the activity of lymphocytes is directly influenced by the insulin level. The effects of insulin may depend on its overall action on metabolic pathways which could explain the general loss of lymphatic tissue in diabetic animals. Some

**Table 1** Suppression of passive transfer of contact sensitivity (CS) by lymphocytes of diabetic animals

| Lymphocytes of diabetic animals, specificity and treatment | CS reaction ( $\times 10^{-3}$ cm) |                |
|------------------------------------------------------------|------------------------------------|----------------|
|                                                            | PCL                                | OX             |
| No cells (-ve control)                                     | 1.7 $\pm$ 0.25                     | 2.4 $\pm$ 0.36 |
| No cells (+ve control)                                     | 6.5 $\pm$ 1.04                     | 8.3 $\pm$ 1.33 |
| PCL                                                        | 3.0 $\pm$ 0.63                     | 8.4 $\pm$ 0.87 |
| OX                                                         | 7.2 $\pm$ 0.74                     | 4.9 $\pm$ 0.56 |
| Not sensitised                                             | 7.0 $\pm$ 0.37                     | 8.0 $\pm$ 0.60 |
| PCL, purified T cells                                      | 2.2 $\pm$ 0.41                     | ND             |
| PCL, anti-Thy 1 treated                                    | 7.8 $\pm$ 0.51                     | ND             |
| OX, purified T cells                                       | ND                                 | 4.0 $\pm$ 0.34 |

Normal CBA recipients were injected with  $4 \times 10^7$  cells of normal animals sensitised with PCL or OX 4 days previously, mixed with  $2 \times 10^7$  cells from diabetic animals which had (or had not) been sensitised with either PCL or OX 4 days before transfer. In some experiments cells from hyperglycaemic mice were purified on nylon wool columns (purified T cells) or treated with anti-Thy 1 serum and C' before being injected to the hosts. This serum killed >90% nylon column purified T cells. Mice were challenged on the ears with the homologous hapten. Each group consisted of six to eight mice. Similar results were obtained with DBA/2 mice. Results are given as mean  $\pm$  s.d. +ve control refers to animals which received immune normal cells only; -ve control refers to mice which received no injection of cells (nonspecific ear swelling).

selectivity of insulin action, however, argues against this simple scheme. It has been found that insulin augments the *in vitro* cytotoxicity of sensitised lymphocytes<sup>13</sup> and insulin receptors have been demonstrated on cytotoxic lymphocytes<sup>14</sup> as well as on cells activated with concanavalin A<sup>15</sup>. The majority of blood lymphocytes do not express demonstrable insulin receptors which suggests that subsets of T lymphocytes may differ in their expression and thus may be selectively sensitive to insulin deficiency.

A large body of data has accumulated which suggests that cytotoxic effector cells and suppressor T lymphocytes share several characteristics<sup>16,17</sup>, including the expression of hormone receptors<sup>14</sup>, which are not shared by helper cells. If these similarities extend to insulin receptors we would then postulate a direct effect of the lack of insulin on the expression of suppressor cell activity. Since the lack of insulin increases suppressor cell activity, as demonstrated above, our results could suggest that the presence of insulin is an important factor in damping down normal suppressor cell activity. This postulate would predict that subjects with deficient levels of insulin would be more susceptible to infection because of hyperactivity of their suppressor cells. However, since the activation of suppressor cells has been shown to be the result of a complex series of interactions between T cell sets and since it has also been shown that the activity of cells outside of the suppressor circuit can influence the levels of suppression which become manifest after immunisation<sup>18</sup>, our results do not indicate directly that the suppressor cell itself is the target of insulin mediated control. Further studies using antisera which define functionally distinct T cell subsets are required before we can definitely say how the lack of insulin leads to increased suppressor cell activity. We are also attempting positive selection studies, using insulin columns to directly test the nature of the cells which binds to the columns. The fact that insulin has been shown to increase the activity of sensitised killer lymphocytes<sup>2,7</sup>, and that its lack appears to increase the level of suppressor cell activity should not be taken as evidence that the putative insulin receptors of these two sets of T cells are in any way different. The possibility that a single agonist can have two opposing effects depending upon dose, should be taken into consideration. The observations, however, that discrete subpopulations of T lymphocytes carry receptors for various hormones (for example, growth hormone<sup>19</sup> or histamine and adrenaline<sup>20</sup>) make our hypothesis of selective expression of insulin receptor tenable and testable.

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## Phototransduction in the fly compound eye exhibits temporal resonances and a pure time delay

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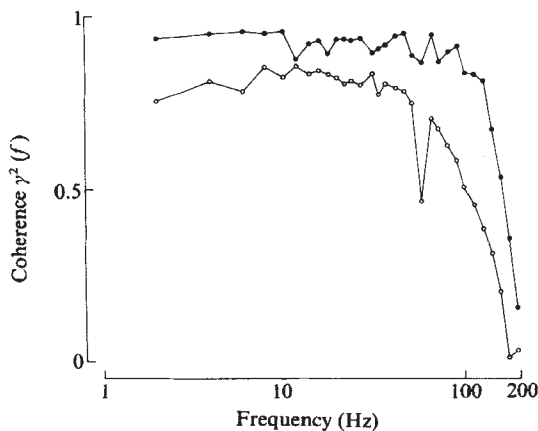
Photoreceptor cells in both vertebrates and invertebrates respond to a flash of light with a slow graded change in membrane potential, which is generally depolarising in invertebrates and hyperpolarising in vertebrates. Although some of the early photochemical and biochemical stages of the transduction process have been elucidated in both cases<sup>1,2</sup>, these reactions are fast compared with the time course of the electrical response, which is typically hundreds of milliseconds long. To explain this slow response in the eye of *Limulus*, Fuortes and Hodgkin<sup>3</sup> proposed a mechanism in which the light signal passes through a cascade of simple low pass filters. The model was later defined more specifically in terms of a chain of chemical reactions linked together through products and reactants<sup>4</sup>, and has been used with small modifications and different numbers of stages to account for the behaviour of various vertebrate and invertebrate photoreceptors including the fly compound eye<sup>5,6</sup>. I have now obtained evidence that phototransduction in the fly in small signal conditions involves underdamped resonance behaviour and a significant pure time delay, neither of which can be accounted for by the conventional cascade model.

The frequency response function is an alternative means of characterising the dynamic behaviour of a photoreceptor, and yields an identical description to that given by the response to a brief flash of light if the photoreceptor behaves linearly. In practice the frequency response method is more useful than the flash method because it allows a photoreceptor to be examined in small signal conditions with relatively small light fluctuations where behaviour is linear. When the white noise stimulation technique is used to measure the frequency response function it is possible to characterise a photoreceptor very efficiently and simultaneously to measure the linearity and signal to noise level of the system through the coherence function<sup>6</sup>.

In the frequency domain the long-delayed response of photoreceptors becomes a phase lag which increases with frequency and there is also a substantial amplitude attenuation with increasing frequency. The Fuortes and Hodgkin model predicts these effects but also predicts a maximum phase lag asymptote the value of which is given by  $90^\circ$  multiplied by the number of stages in the cascade. This asymptotic phase lag has never been demonstrated clearly and most published frequency response functions for photoreceptors show a phase lag which is



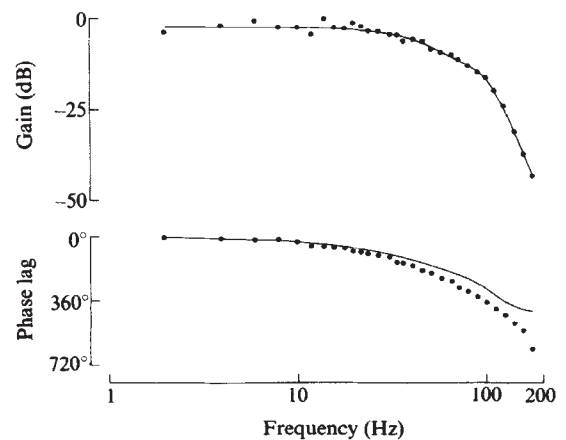
unbounded with increasing frequency. In previous investigations of the fly photoreceptor system<sup>6,7</sup> we tried to find a phase lag asymptote and tentatively suggested that it occurs at a lag of  $450^\circ$ , characteristic of a five-stage cascade. However, examination of the coherence functions which we obtained for this preparation reveals that in the frequency range where the asymptote should appear, which is above 100 Hz in the fly, the signal to noise ratio is so low that there is little statistical confidence in the measurements. Because this problem arises from the inherent noise level of the photoreceptor, the same difficulty will apply to the flash response measurement, but it will be the accuracy of the most rapidly changing regions of the response which are unreliable.



**Fig. 1** Coherence function,  $\gamma^2(f)$ , for phototransduction in a fly photoreceptor cell. The open circles are from an experiment conducted with the conventional white noise technique using 25 sequences of noise, each lasting 512 ms. The input and output data were sampled at 1-ms intervals and processed using the fast Fourier transform. The filled circles are from the same experiment but with each of the 25 sequences being presented 10 times and the average response taken. The improvement in signal to noise ratio may be quantified by use of the expression  $\gamma^2(f) = 1/(1 + N(f)/S(f))$ , where  $S(f)$  is the signal power and  $N(f)$  is the noise power at frequency  $f$ <sup>11</sup>. Applying this equation at 100 Hz gives more than a fivefold improvement in signal to noise power ratio. Solid lines are drawn through the data points for visual clarity. The large dip in the unaveraged data is at 60 Hz, the a.c. power frequency, and represents unavoidable experimental pick up. It is also reduced substantially by the averaging process because it is uncorrelated with the stimulus.

I repeated the frequency response function measurements on the fly photoreceptor system using an averaging technique to improve the signal to noise ratio. The technique consists of stimulating the eye with repeated bursts of pseudo-random noise patterns of light intensity while averaging the responses to identical patterns. This averaging reduces the uncorrelated noise from the response and the averaged signal is processed as if it were the response to a single presentation of the random input. The experiments were conducted on the R1-6 photoreceptors of the compound eye of *Phormia regina* using a green light-emitting diode with peak output at 560 nm. Intact flies were mounted in wax and intracellular recordings of membrane potentials were made by glass microelectrodes filled with 3 M KCl and having resistances in the range 50–100 M $\Omega$ . The microelectrode was advanced through the back of the eye and a platinum ground electrode was placed in the thorax. Details of the experimental procedures and the averaging technique are given elsewhere<sup>8,9</sup>.

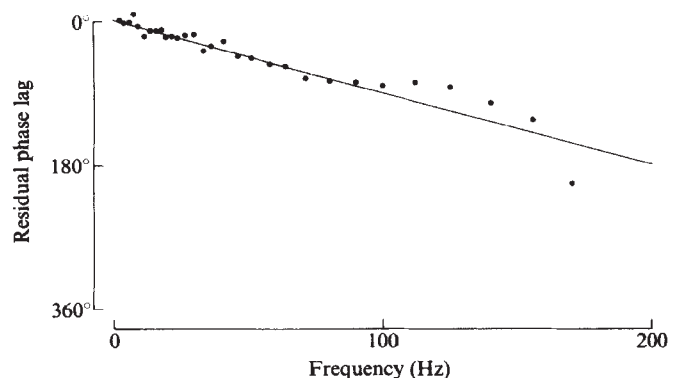
Figure 1 shows a comparison of the coherence function measured for a photoreceptor with and without the averaging technique. There is a substantial improvement in the signal to noise ratio, particularly in the critical frequency region of 100–200 Hz where the phase lag asymptote should appear. Figure 2 shows the frequency response function between light fluctua-



**Fig. 2** Frequency response function between light fluctuations and fluctuations in membrane potential in the same photoreceptor cell as for Fig. 1. The mean current through the light emitting diode was 5.6 mA which gives a peak response of about 10 mV in a dark adapted photoreceptor. The mean intensity was modulated to a depth of 75% by the pseudo-random noise stimulus. The solid lines are the predicted response from a linear system consisting of a cascade of two first order poles with time constants of 1.13 and 1.56 ms and two second order poles with time constants of 3.83 and 1.49 ms and damping factors of 0.79 and 0.25, respectively. The phase data points fall progressively below the predicted response with increasing frequency and the difference between these two sets of values is shown in Fig. 3.

tions and membrane potential fluctuations for the same experiment as Fig. 1. The improved signal to noise ratio produces a smooth frequency response function to 200 Hz, which compares favourably with our published data<sup>6,7</sup>.

A low pass frequency response function can be factorised into an expression containing integrating terms,  $(j\omega)^{-1}$ , first order poles,  $(1 + j\omega\tau)^{-1}$ , and second order poles  $(1 + 2j\zeta\omega\tau + (j\omega\tau)^2)^{-1}$ , where  $j = \sqrt{-1}$ ,  $\omega$  is natural frequency,  $\tau$  represents time constants, and  $\zeta$  represents damping factors<sup>10</sup>. Integrating terms give rise to a constant phase lag of  $90^\circ$  at all frequencies and so were not considered further because of the reliable low frequency phase asymptote of  $0^\circ$ . The Fuortes and Hodgkin model would produce a series of first order poles, with each stage contributing a single time constant. Second order poles represent underdamped or resonant behaviour for  $\zeta < 1$ , or can be factorised simply into two first order poles for  $\zeta > 1$ . Pure time delay may also be present in a linear system and it produces a phase lag equal to the product of the delay and the frequency, without affecting the gain of the response.



**Fig. 3** Difference between the experimental phase lag of Fig. 2 and the predicted phase lag for the linear system which fits the gain data. The residual phase lag has been plotted on linear axes and fitted with a straight line by linear regression. The slope of the straight line corresponds to a delay of 2.48 ms and the linear regression coefficient is 0.95.

Frequency response functions of the type illustrated in Fig. 2 were fitted by an iterative minimum mean square error technique in which an initial estimate of the number and types of poles and their time constants was made. Then a computer program varied each component up and down in value while computing total square error between the estimate and the experimental data. The varied values producing the smallest error were then used as new estimates while the size of the variation was reduced and the process repeated until the variation was below 1%. The fitting process was applied to the gain portion of the response because this is independent of any delay. Initial attempts to fit the experimental data with first order poles were unsuccessful compared to the accuracy with which the data could be fitted using first and second order poles. The solid line in Fig. 2 is the fit produced by two first order poles and two second order poles. This model also gave the best fit to the data from 12 separate experiments, with average time constants and damping factors of:  $\tau_1 = 0.92$  ms,  $\tau_2 = 1.15$  ms,  $\tau_3 = 3.28$  ms,  $\zeta_3 = 0.88$  and  $\tau_4 = 1.41$  ms,  $\zeta_4 = 0.35$ . The model did not account adequately for the phase data, which always lagged further than the prediction at higher frequencies. Because the only behaviour that could explain this discrepancy is a pure time delay the difference between the predicted and actual phase data was plotted on linear axes and fitted with a linear regression line, as illustrated in Fig. 3. This produced a good fit in all experiments with a mean delay value of 2.85 ms and a mean correlation coefficient of 0.93.

These results show that the linear cascade model is inadequate to account for phototransduction in the fly and a model must be constructed which predicts the underdamped behaviour and the pure time delay.

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## Ca<sup>2+</sup> transmembrane fluxes and nerve growth factor action on a clonal cell line of rat pheochromocytoma

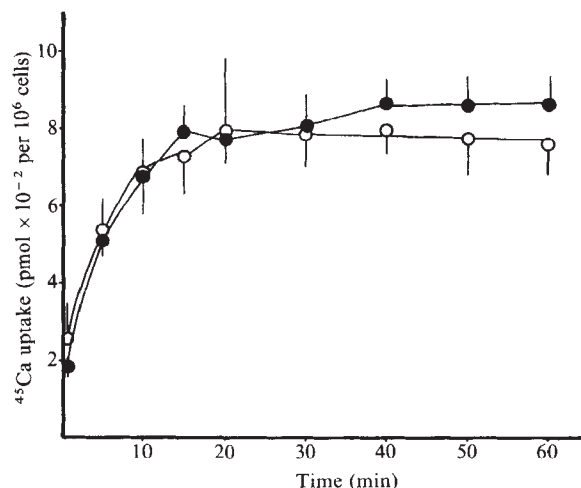
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Nerve growth factor (NGF) is a polypeptide hormone which has a fundamental role in the development and maintenance of sympathetic and some sensory neurones<sup>1,2</sup>. The primary event by which NGF exerts its actions on these neurones probably involves the recognition of specific membrane-located receptors<sup>3–5</sup>. A clonal cell line of rat pheochromocytoma, PC12, responds to NGF by cessation of division and expression of several properties characteristic to mature sympathetic neurones, including neurite outgrowth<sup>6,7</sup>. These cells also possess cell-surface NGF receptors, a fraction of which are occupied at NGF concentrations which elicit NGF-specific responses in these cells (G.L. and E.M.S., unpublished results).

The manner in which binding of NGF to its receptor is translated into the subsequent morphological and biochemical events is not yet known but is being investigated. One possible transducing mechanism which has been suggested involves transient changes of intracellular cyclic AMP levels<sup>8,9</sup>. The divalent cation Ca<sup>2+</sup> has been proposed as a second messenger in the mechanism of action of peptide hormones such as insulin<sup>10,11</sup>, which also initiates its action through membrane-bound receptors. Here, we have investigated the hypothesis that the mechanism of NGF action on PC12 cells may involve modulation of calcium transmembrane fluxes. The evidence shows that NGF has no significant influence on calcium fluxes as measured by uptake and efflux rates of <sup>45</sup>Ca in PC12 cells.

PC12 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 mg l<sup>-1</sup> glucose, supplemented with 10% fetal calf serum and 5% horse serum. For the measurements of calcium fluxes in single cell suspensions of PC12 cells, the culture medium was removed and the cells washed three times with 10 ml of Tris-buffered Gey's solution (TBG), pH 7.4, containing 1.5 mM Ca<sup>2+</sup> and 0.1% bovine serum albumin. The cells were centrifuged for 3 min at 500g, the supernatant discarded and the pellet resuspended in the same buffer at a final concentration of 10<sup>6</sup> cells ml<sup>-1</sup>. Cell viability was evaluated before and after the calcium flux measurements by trypan blue exclusion and was always 85–95%. The cells were preincubated for 40–60 min with gentle shaking at 37 °C in the absence of <sup>45</sup>Ca for the uptake experiments or in the presence of <sup>45</sup>Ca for efflux experiments.



**Fig. 1** <sup>45</sup>Ca uptake by PC12 cells. A suspension of PC12 cells (8 ml of a suspension of 10<sup>6</sup> cells ml<sup>-1</sup>) was preincubated with gentle shaking for 40 min at 37 °C in TBG. At the end of this preincubation <sup>45</sup>Ca was added to one half of the sample to give a final specific activity of 30  $\mu$ Ci  $\mu$ mol<sup>-1</sup>, and NGF was added to the remaining half of the sample together with <sup>45</sup>Ca to the same final specific activity. The <sup>45</sup>Ca content of the cells was determined by layering 0.1 ml of the cell suspension on the top of a two-step sucrose gradient (50  $\mu$ l of 0.15 M and 150  $\mu$ l of 0.3 M sucrose in a 400- $\mu$ l Beckman microfuge tube), centrifuging for 30 s in the microfuge, rapidly freezing the tube and cutting off the section containing the cell pellet. The pellet in the tube tip was treated overnight in 0.4 ml of Protosol, scintillation fluid added and the radioactivity determined. Each point represents the mean ( $\pm$ s.d.) of three separate experiments. ●, Control cells; ○, cells incubated with 3 nM NGF.

The effect of NGF on <sup>45</sup>Ca uptake was measured on suspensions of PC12 cells (Fig. 1). The uptake reaction was initiated by the addition of <sup>45</sup>Ca alone or with 3 nM NGF. NGF had no effect on either the rate or magnitude of the <sup>45</sup>Ca uptake reaction. Similar results were obtained if NGF (3 nM) was added at the beginning of the preincubation period (data not shown).

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**Table 1**  $^{45}\text{Ca}$  efflux from preloaded PC12 cells attached to a substratum

|          | Calcium preloading period (h) | Experiment | Radioactivity released (d.p.m. $^{45}\text{Ca}$ ml $^{-1}$ ) |                     | <i>n</i> |
|----------|-------------------------------|------------|--------------------------------------------------------------|---------------------|----------|
|          |                               |            | 30 min                                                       | 60 min              |          |
| <i>a</i> | 1                             | 1 Control  | 1,450 ( $\pm 150$ )                                          | 1,590 ( $\pm 130$ ) | 3        |
|          |                               | NGF        | 1,620 ( $\pm 230$ )                                          | 1,740 ( $\pm 180$ ) | 3        |
|          |                               | 2 Control  | 1,060 ( $\pm 120$ )                                          | 1,140 ( $\pm 120$ ) | 4        |
|          |                               | NGF        | 1,110 ( $\pm 40$ )                                           | 1,160 ( $\pm 90$ )  | 4        |
| <i>b</i> | 48                            | Control    | 1,140 ( $\pm 150$ )                                          | 1,280 ( $\pm 120$ ) | 3        |
|          |                               | NGF        | 1,010 ( $\pm 110$ )                                          | 1,100 ( $\pm 80$ )  | 3        |

*a*, PC12 cells (log phase) were washed with TBG as described in the text and  $3 \times 10^6$  cells were plated onto polylysine-treated 60-mm tissue culture dishes.  $^{45}\text{Ca}$  was added immediately to a final specific activity of  $1.3 \mu\text{Ci } \mu\text{mol}^{-1}$  and cells were preincubated for 1 hr at  $37^\circ\text{C}$ . The cells were rapidly washed three times in TBG or in TBG containing 3 nM NGF, 4 ml of the appropriate solution added to the dish and incubation carried out at  $37^\circ\text{C}$ . After 30 or 60 min, 0.1-ml aliquots of the medium were taken in quadruplicate and radioactivity determined. In controls where there were no cells in the dish  $\sim 100$  c.p.m. ml $^{-1}$  were released from the dish. The data are presented as the mean of *n* samples; the figures in parentheses are standard deviations and *P* values were determined by Student's *t*-test. *b*, PC12 cells were grown (log phase) in Dulbecco's modified Eagle's medium to which  $1.1 \mu\text{Ci } \mu\text{mol}^{-1}$   $^{45}\text{Ca}$  was added 48 h before the cells were collected. The cells were removed from the tissue culture dish in the TBG containing the same concentration of  $^{45}\text{Ca}$ , washed and then replated on polylysine-treated tissue culture dishes (60 mm,  $3 \times 10^6$  cells per dish) in the same medium containing  $^{45}\text{Ca}$  and preincubated for 1 h at  $37^\circ\text{C}$ . The cells were then rapidly washed three times with calcium-free TBG or calcium-free TBG containing 3 nM NGF and 4 ml of this medium added to the dish. After 30 or 60 min, 0.1-ml aliquots of the medium were taken in quadruplicate and radioactivity determined.

The efflux of  $^{45}\text{Ca}$  from suspensions of PC12 cells was measured after preloading the cells with  $^{45}\text{Ca}$  for short (40 min) and long (25 h) periods. The efflux of  $^{45}\text{Ca}$  from PC12 cells after a short preloading period is shown in Fig. 2. The exposure of the cells to 3 nM NGF produced no significant change in the rate of  $^{45}\text{Ca}$  efflux. If the cells were preloaded for 25 h with  $^{45}\text{Ca}$ , similar results were obtained when tested with 5 nM NGF or as much as 300 nM highly purified 7S NGF (ref. 12, data not shown). Both uptake and efflux studies were carried out with confluent cultures as well as those during log phase growth and identical results were obtained.

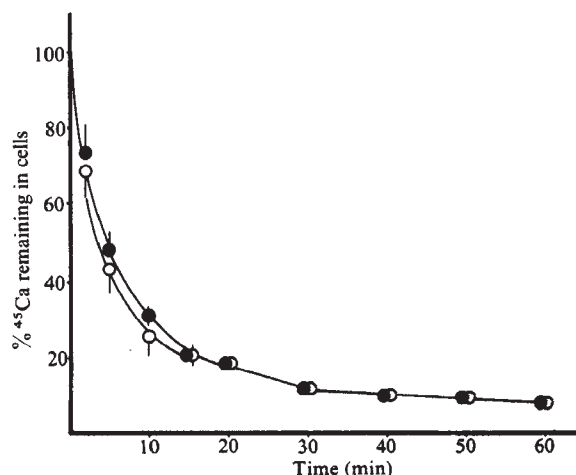
The efflux of  $^{45}\text{Ca}$  from PC12 cells attached to tissue culture dishes was also investigated, as cell membrane-mediated events may be influenced by attachment of cells to a substratum. When PC12 cells (log phase) were preincubated with  $^{45}\text{Ca}$  for 1 h on polylysine-treated tissue culture dishes (60 mm) and the efflux measured in the presence or absence of NGF (3 nM), no difference in the magnitude of the loss of  $^{45}\text{Ca}$  was detected 30 or 60 min later (Table 1). Similarly, if the cells were preincubated for 48 h with  $^{45}\text{Ca}$  and the efflux into calcium-free medium measured, NGF (3 nM) had no significant effect.

These results show that NGF has no demonstrable effect on transmembrane calcium fluxes, when measured with cells either in suspension or attached to a substratum. Efforts to alter intracellular calcium concentrations by addition of the calcium ionophore A23187 were unsuccessful because of the cytotoxic effects of the drug on PC12 cells in serum-free medium. When A23187 was tested in the presence of serum it had no effect on the NGF-mediated morphological differentiation. However, the binding of the drug to serum lipids rather than to the cell is a possibility which cannot be ruled out.

Schubert *et al.*<sup>13</sup> have recently suggested that NGF initiates its action on PC12 cells by causing a transient increase in intracellular cyclic AMP followed by a mobilisation and release of intracellular calcium stores. This conclusion was based on experiments which showed a small increase in the efflux of  $^{45}\text{Ca}$  from NGF-treated cells compared with untreated cells<sup>13</sup>. The experiments reported here do not show an NGF-mediated alteration of  $^{45}\text{Ca}$  efflux from cells in suspension when either low concentrations (3 nM) of NGF or high concentrations (300 nM) of 7S NGF were added. Moreover, the data shown in Table 1*b* were obtained in the same conditions as described by Schubert *et al.*<sup>13</sup> except that the NGF concentration was substantially lower. The lower NGF concentrations were chosen because the PC12 NGF receptors are fully saturated at 3 nM NGF (G.L. and E.M.S., unpublished observations), whereas half-maximal stimulation of neurite outgrowth occurs at 0.01 nM NGF (ref. 14). The binding of NGF to its cell-surface receptor does not

require calcium<sup>15</sup>. It is noteworthy that Schubert *et al.*<sup>13</sup> used 800 biological units ml $^{-1}$  of the 7S NGF complex ( $\sim 300$  nM), resulting in NGF concentrations several orders of magnitude above the physiologically effective concentrations of NGF for these cells. Even when tested at this concentration we were unable to detect any NGF-mediated alteration of  $^{45}\text{Ca}$  efflux from suspensions of PC12 cells.

The hypothesis that NGF causes a rapid but transient increase in intracellular cyclic AMP preceding the calcium mobilisation



**Fig. 2**  $^{45}\text{Ca}$  efflux from PC12 cells. The time course of the  $^{45}\text{Ca}$  efflux was measured after a suspension of PC12 cells ( $10^6$  cells ml $^{-1}$ ) had been preincubated at  $37^\circ\text{C}$  in TBG containing  $5 \mu\text{Ci } \mu\text{mol}^{-1}$   $^{45}\text{Ca}$ . After 60 min, 0.1-ml aliquots were taken and the cellular  $^{45}\text{Ca}$  content determined as described in Fig. 1 legend. The radioactivity in the cells at this time was defined as 100%. The suspension was divided into two halves, each half centrifuged at 500g for 30 s, the supernatants discarded and the pellets resuspended with trituration in the same buffer but without  $^{45}\text{Ca}$ . One half of the sample received no addition, and 3 nM NGF was added to the other half. The amount of  $^{45}\text{Ca}$  remaining in the cells was evaluated by sampling 0.1-ml aliquots in triplicate at various times after transfer to the  $^{45}\text{Ca}$ -free buffer. ●, Control cells; ○, cells incubated with 3 nM NGF. Each point represents the mean ( $\pm$ s.d.) of a series of three experiments, with each experiment consisting of triplicate samples. In each of the three individual experiments no significant difference between control and NGF-treated samples was observed.

in PC12 cells has also been questioned. Hatanaka *et al.*<sup>16</sup> could not demonstrate any alteration of intracellular cyclic AMP levels by NGF, in contrast to the findings of Schubert *et al.*<sup>9,13</sup>. The relationship of intracellular calcium concentrations and cellular differentiation is unclear<sup>17</sup>, but it is possible that calcium may be involved in certain aspects of differentiation. However, in the light of the data presented here and those of others<sup>16</sup>, the hypothesis that NGF action is mediated through cyclic AMP-dependent calcium mobilisation must be viewed with caution.

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## Oscillation of $[Ca^{2+}]_i$ -linked $K^+$ conductance in bullfrog sympathetic ganglion cell is sensitive to intracellular anions

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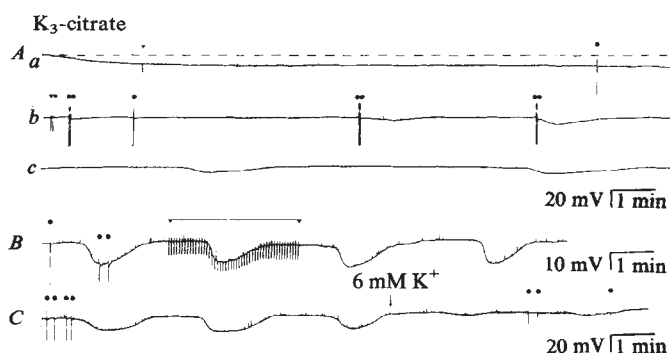
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The intracellular free  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) regulates the  $K^+$  conductance ( $G_K$ ) of the many types of cell membrane<sup>1–11</sup>. The  $Ca^{2+}$  influx during an action potential activates this  $[Ca^{2+}]_i$ -linked  $G_K$  in most neurones<sup>2–4,8–11</sup>. In caffeine-treated sympathetic ganglion cells, however,  $Ca^{2+}$  released from an intracellular  $Ca^{2+}$  reservoir site analogous to the sarcoplasmic reticulum (SR) of the muscle (see ref. 12) causes activation of the  $G_K$ , which results in slow oscillatory hyperpolarisations (caffeine hyperpolarisation, C-hyperpolarisation)<sup>11</sup>. Such a release of  $Ca^{2+}$  linked to the  $G_K$  of the membrane seems important for understanding the role of the intracellular organelles in the control of membrane activities of a neurone. We report here the mechanism of the slow oscillatory hyperpolarisations recorded from the bullfrog sympathetic ganglion cell in Ringer solution. It is found that these hyperpolarisations are generated by a  $[Ca^{2+}]_i$ -linked  $G_K$  system and are highly sensitive to anions in an intracellular recording electrode, probably to intracellular anions.

Experimental procedures for intracellular recording from B-type neurones of paravertebrate sympathetic ganglia of the bullfrog (*Rana catesbeiana*) have been described previously<sup>11,13,14</sup>. Most experiments were carried out using microelectrodes filled with 1 M  $K_3$ -citrate (tip resistance 20–60 M $\Omega$ ). In some experiments, electrodes were filled with 0.3 M

$K_2$ -sulphate, 3.0 M K-acetate or 3 M KCl (tip resistances 30–100 M $\Omega$ ). Experiments were carried out at room temperature (20–25 °C).

When the ganglion cell was impaled with an electrode filled with 1 M  $K_3$ -citrate in Ringer, the resting membrane potential was relatively low (–40 to –50 mV), probably due to penetration injury<sup>15</sup>. Within a few minutes, the membrane began to hyperpolarise (repolarise), with an increase in the membrane resistance. In 10–20 min, the resting membrane potential and resistance reached a relatively high constant value (Fig. 1Aa, b). In this condition, the generation of an action potential was followed by a slow hyperpolarisation with a notable delay in many of the cells impaled (26 cells out of 38) (Fig. 1Ab). After a further 5–10 min, slow rhythmic hyperpolarisations appeared spontaneously in most cells (23 cells out of 38; Figs 1Ac, B, C and 2Cc). The amplitude ( $7.3 \pm 0.8$  mV (s.e.),  $n = 23$ ) and time course (half-duration  $1.6 \pm 0.4$  min) of these transient hyperpolarisations and the rate of their appearance (one every  $6.4 \pm 0.9$  min,  $n = 23$ ) remained almost constant as long as the resting potential was unchanged. Because, as will be described below, these hyperpolarisations seen in Ringer were very sensitive to intracellular anions, they will be called hereafter anion-sensitive hyperpolarisations (A-hyperpolarisations).

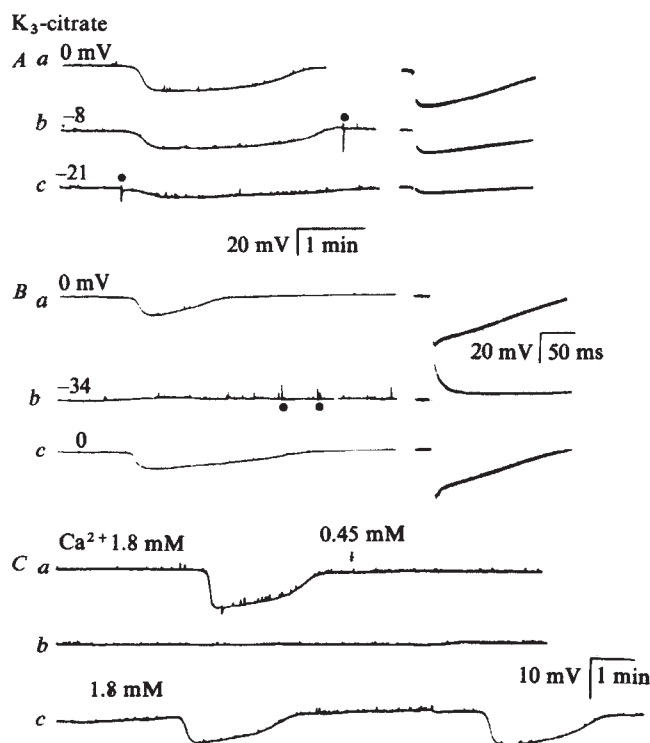


**Fig. 1** Slow rhythmic hyperpolarisations (A-hyperpolarisations) recorded from three different cells. An action potential was induced by antidromic stimulation at times indicated by a closed circle (A–C). A hyperpolarising current pulse (0.3 nA, 250 ms) was applied through a recording electrode at times indicated by a triangle (A) or the current pulses (0.2 nA, 250 ms) were applied repetitively for the period indicated by the two triangles (B). A, Example of the time course of the membrane potential change after the penetration of the ganglion cell with an electrode filled with 1 M  $K_3$ -citrate. All the records (a–c) are continuous. The recording electrode was inserted immediately before the beginning of the record a. The spike component of the action potential is not shown (this is also true of the remaining figures where action potentials are shown). Note the appearance of an A-hyperpolarisation following the after-hyperpolarisation of an action potential and spontaneously occurring A-hyperpolarisations. B, The reductions of the membrane resistance and the amplitude of the after-hyperpolarisation of an action potential during the generation of an A-hyperpolarisation. C, Effects of an increase in the  $K^+$  concentration in Ringer on A-hyperpolarisations.

The membrane resistance decreased dramatically during the generation of an A-hyperpolarisation (Fig. 1B). Raising the external  $K^+$  concentration (6 mM) markedly reduced the amplitude of A-hyperpolarisations, although it depolarised the membrane only slightly (Fig. 1C). Conditioning membrane hyperpolarisations decreased the amplitude of the A-hyperpolarisation (Fig. 2A) and seemed to be reversed at a level at which the after-hyperpolarisation of an action potential was reversed (Fig. 2B). Thus, A-hyperpolarisations are apparently induced by an increased  $G_K$  of the membrane.



As in the case of C-hyperpolarisations<sup>11,13,14</sup>, A-hyperpolarisations were extremely sensitive to the external  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_o$ ). Lowering  $[\text{Ca}^{2+}]_o$  completely abolished A-hyperpolarisations (four cells) or decreased the rate of their appearance (three cells) in 3–5 min after the solution change (Fig. 2C), whereas the frequency of their appearance increased when  $[\text{Ca}^{2+}]_o$  was raised. These effects of  $[\text{Ca}^{2+}]_o$  were reversible. Furthermore, A-hyperpolarisations were sensitive to low temperature and inhibited by dantrolene Na (6.4  $\mu\text{M}$ ), as observed in the C-hyperpolarisation<sup>11,13</sup>. Thus, A-hyperpolarisations seem to be generated by a mechanism similar to that for C-hyperpolarisations, that is, increases in the  $G_K$  due to periodic rises in  $[\text{Ca}^{2+}]_i$ .



**Fig. 2** A, B, Effects of conditioning membrane hyperpolarisations on the A-hyperpolarisation (left records) and the after-hyperpolarisation of an action potential (right records) in two cells. The level of membrane hyperpolarisation shown in the left side corresponds to both A-hyperpolarisations and after-hyperpolarisations of action potentials. An action potential was evoked by antidromic stimulation at times marked by closed circles in the left record of Ab, Ac and Bb. Action potentials are not shown in the left records of Aa, Ba and Bc. C, Effects of a reduction in the extracellular  $\text{Ca}^{2+}$  concentration in Ringer on A-hyperpolarisations. All the records are continuous. Note the disappearance of A-hyperpolarisations at a reduced  $\text{Ca}^{2+}$  concentration.

There is, however, a definite difference between the experimental conditions for recording A- and C-hyperpolarisations. The former were recorded by impaling a cell with an electrode filled with 1 M  $\text{K}_3$ -citrate in Ringer, whereas the latter were recorded with a 3 M KCl-filled electrode in a solution containing caffeine. This would imply that the species of an anion leaked out of the electrode into the cytoplasm, would determine the requirement for caffeine in the generation of oscillatory hyperpolarisations. Indeed, a very low dose of caffeine (0.1–1.0 mM) unable to induce C-hyperpolarisations augmented the amplitude and frequency of the A-hyperpolarisation, indicating that there was an increase in the apparent sensitivity of the ganglion cell to caffeine, when it was impaled with a  $\text{K}_3$ -citrate electrode.

**Table 1** Effects of various anions in a recording electrode on the appearance of slow rhythmic hyperpolarisations at different caffeine concentrations

|                                | Caffeine (mM) |              |              |
|--------------------------------|---------------|--------------|--------------|
|                                | 0.5           | 1.0          |              |
| $\text{K}_3$ -citrate (1.0 M)  | 21/23 (91%)   | 25/25 (100%) | —            |
| K-acetate (3.0 M)              | 11/16 (69)    | 14/18 (78)   | 18/18 (100%) |
| $\text{K}_2$ -sulphate (0.3 M) | 8/12 (67)     | 11/15 (73)   | 15/15 (100)  |
| KCl (3.0 M)                    | 4/18 (23)     | 11/22 (50)   | 22/22 (100)  |

Numerators are the number of cells in which rhythmic hyperpolarisations were observed, and denominators are the number of cells examined. In the experiments using electrodes filled with K-acetate,  $\text{K}_2$ -sulphate and KCl, the cells which showed rhythmic hyperpolarisations at a caffeine concentration of 3 mM were studied. When caffeine was absent rhythmic hyperpolarisations were recorded only in the cells which were penetrated with an electrode filled with 1 M  $\text{K}_3$ -citrate.

The ability of various anions to generate slow oscillatory hyperpolarisations was tested by penetrating a cell with an electrode filled with K-salts of various anions, and observing for each electrode the number of the cells which exhibited slow oscillatory hyperpolarisations at a given concentration of caffeine (Table 1). The order of favourable anions was citrate > acetate and/or > sulphate > Cl.

There may be several interpretations for this order. First, assuming that both A- and C-hyperpolarisations are generated by the intracellular release and uptake cycle of  $\text{Ca}^{2+}$  at a  $\text{Ca}^{2+}$  storage site<sup>11</sup>, this order of anions may indicate their ability to release  $\text{Ca}^{2+}$  from the storage site like caffeine; citrate would be most effective, and  $\text{Cl}^-$  would have no effect. The latter case would thus require caffeine. Second, the order of these anions may reflect depressant actions on the  $\text{Ca}^{2+}$  release mechanism in the ganglion cell. Endo<sup>16</sup> reported that a sustained treatment of the SR with a low concentration of  $\text{Cl}^-$  inactivated the release of  $\text{Ca}^{2+}$ . As the  $\text{Ca}^{2+}$  storage site in the ganglion cell seems to resemble the SR in its properties<sup>11,13</sup>,  $\text{Cl}^-$  leaked out of an electrode into the cytoplasm may depress the  $\text{Ca}^{2+}$  release mechanism in the ganglion cell. This  $\text{Cl}^-$  action may be antagonised by caffeine. If so, citrate may be inert and A-hyperpolarisations could be a physiological phenomenon. We do not yet know which of these possibilities is operative. In any case, it is quite clear that the species of intracellular anions in the ganglion cell markedly affects the mechanism of the slow oscillatory hyperpolarisations.

It is unlikely that the present findings are the result of a chelating action of citrate because the estimated intracellular concentration of citrate was not high enough to produce a chelating action, and because such a chelating action would have blocked A-hyperpolarisations<sup>11,13,14</sup>.

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## Glucocorticosteroids modulate transmitter choice in developing superior cervical ganglion

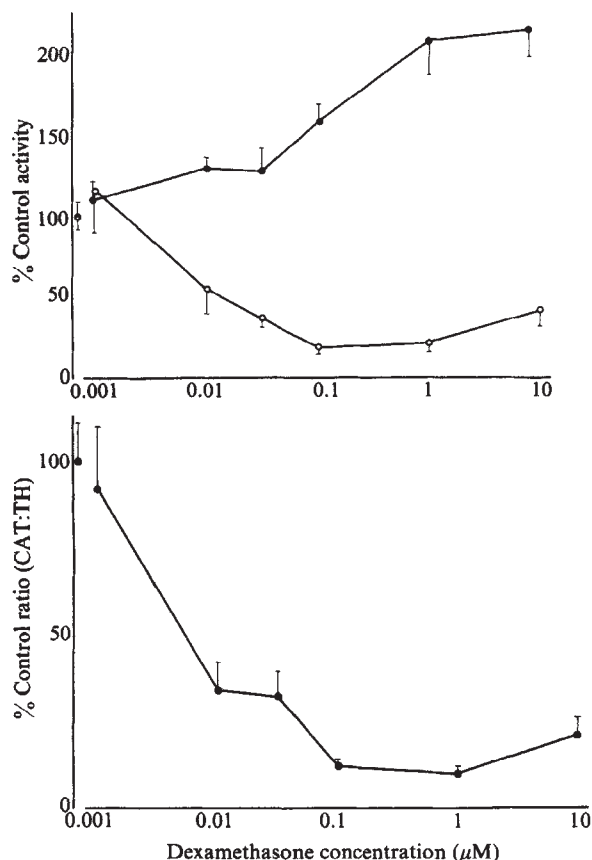
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*In vivo* the superior cervical ganglion (SCG) is adrenergic with less than 5% of neurones expressing cholinergic properties<sup>1</sup>. *In vitro* these neurones can develop either an adrenergic or cholinergic phenotype, depending on the nature of the culture conditions<sup>2</sup>: when immature SCG neurones are cultured in the virtual absence of non-neuronal cells they develop adrenergic properties, synthesising noradrenaline (NA) and a negligible amount of acetylcholine (ACh)<sup>3,4</sup>; when ganglionic non-neuronal cells are present the neurones develop a cholinergic phenotype, characterised by increased ACh synthesis and a corresponding decrease in NA synthesis<sup>5</sup>. If ganglionic non-neuronal cells release a factor which induces cholinergic properties *in vitro*, why is the SCG not cholinergic *in vivo*? *In vitro* there are none of the presynaptic, postsynaptic, or circulatory hormonal influences normally present in neuronal development. Depolarisation of SCG neurones *in vitro* prevents the development of cholinergic properties, suggesting that the preganglionic input *in vivo* may maintain the adrenergic nature of the SCG<sup>6</sup>. However, as prevention of depolarisation of neurones *in vivo* by preganglionic nerve section does not alter the adrenergic character of the SCG, influences other than the preganglionic input must be involved in preventing the development of cholinergic properties *in vivo*<sup>7</sup>. Circulatory glucocorticosteroids modulate the induction of neurotransmitter synthetic enzymes in the SCG<sup>8,9</sup> and have been reported to change the expression of adrenergic and cholinergic properties in a pheochromocytoma cell line<sup>10</sup>. We present here results suggesting that glucocorticosteroids are involved in specifying the transmitter type used in the SCG.

SCG from 3-day-old rats were cultured on collagen-coated plastic multitrays (Linbro 76-033-05) in Dulbecco modified Eagle's medium containing 50 mM HEPES, 100 U ml<sup>-1</sup> penicillin G, 10% inactivated horse serum, 1 µg ml<sup>-1</sup> β-nerve growth factor (βNGF) and varying concentrations of dexamethasone (DXM) or corticosterone. After 14 days incubation choline acetyltransferase (CAT) and tyrosine hydroxylase (TH) activity were determined by previously described methods<sup>12,13</sup>.

DXM antagonised the conversion of the SCG from an adrenergic to a cholinergic phenotype *in vitro*, resulting in a dose-related increase in the activity of TH and a corresponding



**Fig. 1** Effect of dexamethasone on enzyme activities in cultured superior cervical ganglia. *a*, Effect on tyrosine hydroxylase (●) and choline acetyltransferase (○) levels; *b*, effect on the ratio of choline acetyltransferase to tyrosine hydroxylase activity. Activities are expressed as a percentage of the activity of control ganglia grown in the absence of dexamethasone. Control values were TH:  $62 \pm 5$  pmol h<sup>-1</sup> per ganglion; CAT:  $0.76 \pm 0.07$  nmol h<sup>-1</sup> per ganglion and CAT:TH  $12.2 \pm 1.3$ . Points are means  $\pm$  s.e.m. for 12, 6, 6, 12, 12, 12 ganglia respectively. All ganglia were cultured for 14 days and the medium was changed every 4 days.

decrease in CAT activity (Fig. 1a). The CAT:TH ratio, a sensitive measure of the degree of cholinergic character, decreased with increasing DXM concentration, reaching a minimum at  $10^{-7}$  M DXM (Fig. 1b).

The naturally occurring glucocorticosteroid in rats is corticosterone. At the physiological plasma level for neonatal rats ( $1 \mu\text{M}$ )<sup>14</sup>, this steroid was similar to DXM in its ability to alter the levels of CAT and TH. The ratio of CAT:TH in these cultures is comparable to the CAT:TH ratio observed in decentralised ganglia *in vivo* (Table 1).

These results indicate that physiological levels of glucocorticosteroids can influence the choice of transmitter type of the developing SCG *in vitro*. The glucocorticosteroids may be acting on (1) the non-neuronal cells to cause either a decrease in the production or liberation of a factor inducing cholinergic properties, (2) directly on the neurones to alter their responsiveness to such a factor or (3) selective destruction of cholinergic neurones. Irrespective of whether glucocorticosteroids act directly or indirectly on neurones it is likely that during the development of the SCG *in vivo* circulating levels of corticosterone are in part responsible for the adrenergic phenotype of the ganglion, despite the presence of non-neuronal cells which *in vitro* cause the SCG to become primarily cholinergic.

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**Table 1** Effect of glucocorticoids on the ratio of choline acetyltransferase (CAT) to tyrosine hydroxylase (TH) in cultured rat superior cervical ganglia and comparison with the intrinsic ratio *in vivo*

|                            | CAT:TH         |
|----------------------------|----------------|
| <i>In vitro</i>            |                |
| Control                    | $13.6 \pm 1.8$ |
| Dexamethasone $10^{-7}$ M  | $1.0 \pm 0.2$  |
| Corticosterone $10^{-6}$ M | $0.5 \pm 0.1$  |
| Corticosterone $10^{-5}$ M | $0.0 \pm 0.2$  |
| <i>In vivo</i>             |                |
| Decentralised              | $0.6 \pm 0.1$  |

Superior cervical ganglia from 3-day-old rat pups were decentralised or placed in culture and the activity of TH and CAT determined 14 days later. Ratios are means  $\pm$  s.e.m. for six ganglia.



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## Compact myelin exists in the absence of basic protein in the shiverer mutant mouse

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The myelin sheath is a multilamellar membrane system which surrounds axons in vertebrates and provides the electrical insulation necessary for saltatory nerve impulse conduction. Myelin forms from its cell of origin as a flattened, membrane-bound cytoplasmic process which wraps spirally around the axon; a periodic compact array of membrane pairs is produced from the wrappings as the cytoplasmic contents are extruded, and the external surfaces of membranes become apposed<sup>1,2</sup>. Neurological mutant mice which show myelin abnormalities are useful models for examining the formation, stability and breakdown of myelin. For example, the shiverer mouse carries an autosomal recessive mutation<sup>3</sup> (*shi*)<sup>4</sup> that results in severe myelin deficiency in the central nervous system (CNS)<sup>5,6</sup>, apparently due to a defect in myelin formation<sup>5,6</sup>. The small amount of myelin that does form in the CNS is generally not compacted at its cytoplasmic surfaces<sup>6</sup>, possibly due to the low level of basic protein in shiverer CNS tissue<sup>7</sup>. In the peripheral nervous system (PNS), in contrast, amounts of compact myelin seem to be normal<sup>6</sup>. The coarse tremor and convulsions that begin at about 2 weeks of age in the shiverer are presumably due to the severe CNS deficiency of myelin, as similar neurological signs are shown by other mutants with reduced CNS myelin<sup>8</sup>. Most studies on such mutants have concentrated on those regions of the nervous system which are grossly deficient in myelin<sup>5–10</sup>. In the other regions myelin seems by light microscopy to be normal. At the ultrastructural and molecular level, however, this myelin sometimes shows abnormalities<sup>11–14</sup>, and this has prompted us to examine intensively such myelin in several neurological mutants. For this we have used X-ray diffraction, electron microscopy and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We report here that, of the mutants we have examined so far, the shiverer mouse is unique in showing a striking alteration in myelin protein composition that does not significantly affect the gross morphology and lamellar organisation of the myelin sheath. Our results thus question the proposed role of basic proteins<sup>15–19</sup> in myelin as 'structural cement'.

X-ray diffraction patterns of myelin were obtained from freshly dissected sciatic nerves of adult normal and shiverer mice (Fig. 1a). The patterns extend to  $0.06 \text{ \AA}^{-1}$  and are similar in their fundamental spacings and in the relative intensities of the reflections; this indicates that the packing and molecular

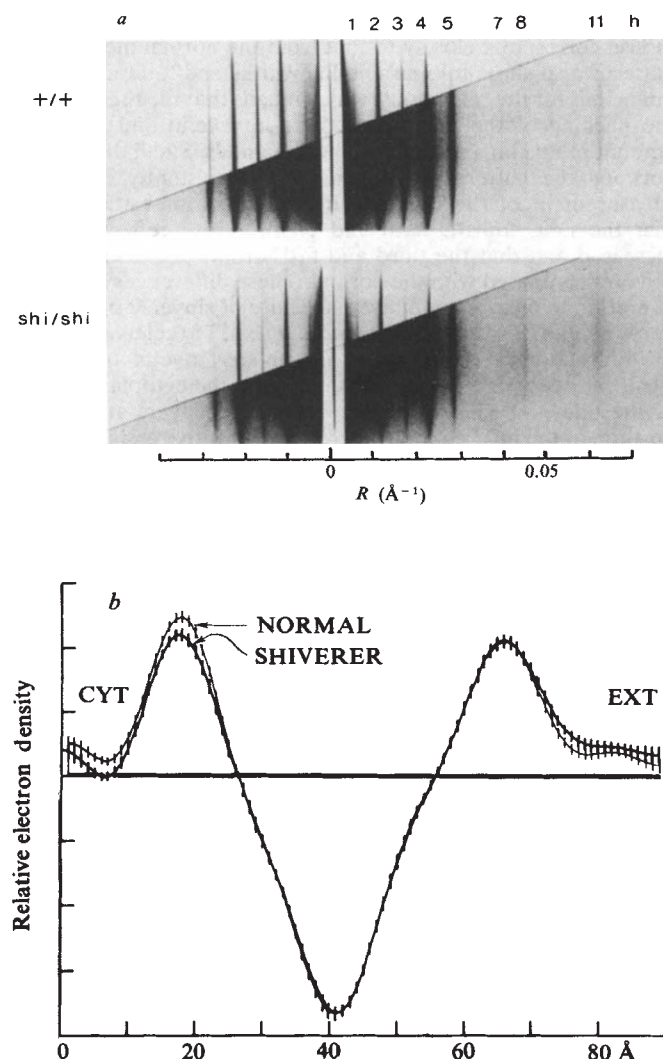
organisation of the membranes in myelin from the shiverer mouse correspond closely to that from the normal mouse. The patterns also show comparable linewidths and total integrated intensities for the reflections, which means that the regularity of the packing of the membrane pairs in myelin and the total amount of myelin are similar in both animals. Small differences between the patterns, however, were consistently observed. Measurement of the normalised diffraction intensities shows that the first, fourth, sixth and eighth order reflections are increased and that the third and fifth orders are decreased in shiverer compared with the normal. These differences reveal an alteration in membrane bilayer structure of shiverer peripheral nerve myelin. Electron density profiles (Fig. 1b) calculated from the diffraction data show that in shiverer myelin there is a significant decrease in electron density on the cytoplasmic side of the bilayer both in the space between bilayers and at the position of the lipid polar head groups. Definitive assignment of these changes in electron density to particular molecular constituents requires chemical characterisation of shiverer peripheral nerve myelin.

Electron micrographs of adult shiverer mouse sciatic nerve show that the myelin is abundantly present as normally compact arrays of membrane pairs (Fig. 2). High magnification electron micrographs of shiverer myelin reveal typical major dense lines and double intraperiod lines which represent, respectively, the appositions of membranes at their cytoplasmic and at their extracellular surfaces. These results confirm the conclusions drawn from the X-ray diffraction measurements that the relative amounts of myelin and the regularity of membrane packing in normal and shiverer mice are similar. Moreover, electron micrographs of shiverer sciatic nerve do not reveal any obvious signs of myelin breakdown such as Schwann cell lysosomes filled with myelin debris or macrophage phagocytosis of the sheath.

The protein composition of myelin in normal and shiverer nerves was analysed with SDS-PAGE. Whole sciatic nerves were dissolved in SDS and electrophoresed in SDS on polyacrylamide gradient slab gels using the discontinuous buffer system of Maizel<sup>20</sup>. The myelin protein bands were identified by co-electrophoresing isolated myelin from normal mouse sciatic nerve and purified myelin proteins. Before examining the shiverer mutant, which is on a hybrid background in our laboratory, we compared the electrophoretic patterns of sciatic nerve proteins from six strains of normal mice (C57BL/6J, DBA/2J, BALB/CWt, C3H/HeJ, CBA/J and AKR/J). The patterns were virtually identical. Therefore, the hybrid background of shiverer would not be expected to produce any variations that might be seen in the banding patterns—variations would be attributable to the mutation.

The gel of normal and shiverer sciatic nerves from animals of different ages (Fig. 3) shows that the major myelin protein,  $P_0$  glycoprotein<sup>21</sup>, is present in comparable amounts at all ages. In contrast, the myelin basic proteins  $P_1$  and  $P_2$  (ref. 21) are present in normal but are absent from shiverer nerves. We also note small differences between shiverer and normal among certain minor protein bands of myelin. We are now examining whether the basic proteins are present transiently in shiverer before 2 weeks of age, during the earliest stages of myelin formation. We have also found that the basic proteins were undetected in adult shiverer sciatic nerve even after loading the gel with twice the usual amount. In trigeminal nerve of the shiverer mouse a similar absence of myelin basic proteins was found. The fact that no additional bands appear in the shiverer gels means that no other proteins are produced which could replace the basic proteins, with the possible exception of small peptides that could pass through the 18% acrylamide portion of the gel and be undetected.

The apparent lack of myelin basic proteins in peripheral nerves of the shiverer mouse might be explained by selective proteolytic activity during preparation for SDS-PAGE. We tested this possibility by three separate procedures. (1) Shiverer sciatic nerves were boiled for 2 min in 2% SDS immediately after dissection to denature proteases. (2) Shiverer sciatic nerves



**Fig. 1** X-ray diffraction from sciatic nerves of normal (+/+) and shiverer (shi/shi) mice. *a*, Diffraction patterns were obtained at room temperature from nerves mounted under tension in thin-walled quartz capillaries containing phosphate-buffered saline (0.9% NaCl, 0.01 M sodium phosphate, pH 7.4). The X-ray source was a fine-line Philips X-ray tube operated at 40 kV, 15 mA. Linear collimation and monochromatisation for CuK $\alpha$  radiation was achieved using a bent nickel-coated optical flat. The X-ray camera had a specimen-to-film distance of 200 mm. Exposure times were 12–20 h using Ilford Industrial G film. Top films and underfilms are presented to show both the strong, low order reflections and the weak, higher orders. The reciprocal space coordinate,  $R$ , is indicated along the bottom and the Bragg or diffraction orders,  $h$ , are numbered along the top. Both diffraction patterns show strong, even orders and weak, odd orders for  $R < 0.03 \text{ \AA}^{-1}$  and show weak, discrete reflections to  $R \approx 0.06 \text{ \AA}^{-1}$ . The most obvious differences between the patterns are the decreased intensities of the third and fifth orders, and the increased intensity of the first order in the shiverer. The spacings and optical densities of the diffraction orders were measured by a Joyce-Loebl MK III C microdensitometer. The repeat period measured for normal sciatic nerve myelin is  $177 \text{ \AA}$  and for shiverer it is  $178 \text{ \AA}$ ; the  $1\text{-\AA}$  difference is less than the variability among different specimens. Measurements of the diffracted intensities were made on sciatic nerves from six normal and two shiverer mice. Integrated peak intensities,  $I(h)$ , were determined by planimetry after background subtraction using a Numonics digitiser interfaced to a PDP 11/05 computer. Structure amplitudes,  $F(h)$ , were calculated from  $(hI(h))^{1/2}$  and were normalised so that  $\sum_h F^2(h)/d = \text{constant}^{37}$ . The final sets of amplitudes that were used to derive the electron density profiles for the normal and shiverer myelin membranes were obtained by calculating a weighted average of the amplitudes for each diffraction order  $h$ . Individual standard deviations of the structure amplitudes were used as the weighting factors. These standard deviations arise from determining peak intensity over background, comparing the integrated intensity from corresponding peaks on either side of the direct beam, and scaling the weak and strong reflections recorded on different films during an exposure. The mean uncertainty of the measurements, calculated from the sum of the standard deviations of the amplitudes divided by the sum of the amplitudes, was about 6%. *b*, Electron density profiles of the myelin membranes were calculated from the structure amplitudes,  $F(h)$ , with phases assigned as previously described<sup>12,38</sup>. The profiles are plotted from the centres of the cytoplasmic fluid spaces (cyt) and extend to the centres of the extracellular fluid spaces (ext) between membrane bilayers; this represents half the membrane pair which constitutes the repeating unit of myelin. The short vertical bars on the density profiles show an uncertainty of 1 s.d. from the mean value of the profile<sup>12</sup>. The profiles were superposed to minimise the differences between the membrane bilayers. Overall, the membranes have similar electron density distributions. However, the shiverer myelin membrane does show a significant decrease in electron density at the polar head group region on the cytoplasmic half of the lipid bilayer. This could be accounted for by a loss of electron dense protein and/or of lipid polar groups. There is also in shiverer myelin a decrease of the average electron density in the cytoplasmic space which might be due to a local decrease in protein concentration.

were homogenised in 2% SDS containing three protease inhibitors ( $10^{-5}$  M leupeptin<sup>22</sup>,  $10^{-4}$  M *o*-phenanthroline<sup>23</sup>,  $10^{-3}$  M phenylmethyl sulphonyl fluoride<sup>24</sup>; Sigma). (3) The sciatic nerves of normal and shiverer mice were homogenised together in 2% SDS; in this mixture myelin basic proteins present in normal nerve would be exposed to any protease in shiverer nerve. Boiling or the use of protease inhibitors had no effect on the gel pattern of shiverer sciatic nerve. Mixing of shiverer nerve with the normal nerve did not alter the amount of basic proteins expected from the normal. We conclude that proteolytic activity does not explain the absence of basic proteins in shiverer peripheral nerve myelin.

Radioimmunoassay (RIA) provides a second, more sensitive method for detecting myelin basic proteins. A quantitative comparison of the levels of  $P_1$  basic protein in normal and shiverer sciatic nerves was made (Table 1); inclusion of the detergent cetyltrimethylammonium bromide in the RIA buffer<sup>25</sup> ensured full expression of this protein. The value for the amount of  $P_1$  basic protein in shiverer, using 1% nerve homogenates, is at the background level of the assay. This indicates at least a 200-fold reduction of basic protein in shiverer relative to normal. Determination of the absolute amount of  $P_1$  basic protein in shiverer would depend on using more concentrated nerve homogenates. Failure to detect this protein in shiverer nerve might be due to inhibition of the antigen–antibody reaction in the RIA by some factor endogenous to shiverer nerves. We tested this possibility by carrying out the assay on a one-to-one mixture of normal and shiverer nerve homogenates. The level of  $P_1$  basic protein measured was half the amount in normal nerve that would be expected if there were no interference with

the antigen–antibody reaction. The RIA also indicates that immunoreactive fragments of  $P_1$  basic protein are not present in shiverer nerve. We intend to quantitate the parallel lack of  $P_2$  basic protein from shiverer peripheral nerves using RIA.

We have found that shiverer peripheral nerve myelin lacks basic proteins but that the lamellar arrangement of membranes—the repeat period and the widths of the cytoplasmic and extracellular spaces—are the same as in normal myelin. At the level of the individual myelin membrane bilayers, however, we find a small decrease in electron density of the polar head group region in the cytoplasmic half of the bilayer, which could be accounted for by the absence of basic proteins from that region. This decrease might also be explained by a reduced proportion of phosphate-containing lipids, a possibility that is now being examined.

**Table 1** Radioimmunoassay of  $P_1$  basic protein in mouse sciatic nerve

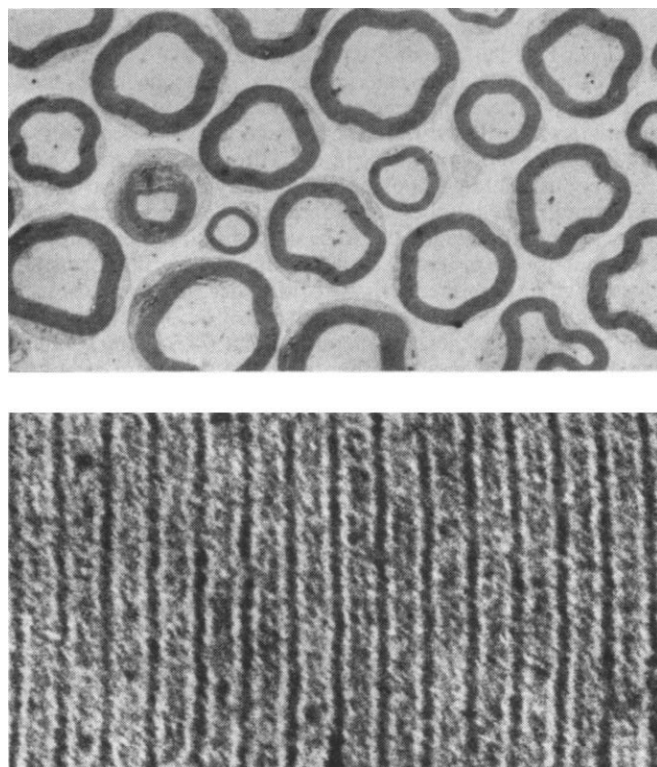
|          | $\mu\text{g } P_1 \text{ per mg wet weight}$ |
|----------|----------------------------------------------|
| Normal   | $4.05 \pm 0.46$                              |
| Shiverer | $0.019 \pm 0.015$                            |

1% (w/v) homogenates of sciatic nerves were made in RIA buffer containing 0.2 M Tris-acetate (pH 7.3), 1% cetyltrimethylammonium bromide, 1 mg ml<sup>-1</sup> calf thymus histone, 0.5% (w/v) calf serum and 0.02% sodium azide. The homogenates were centrifuged to remove connective tissue and 10- $\mu\text{l}$  aliquots of the supernatants, diluted 1:100 with RIA buffer for the normal and undiluted for the shiverer, were used for the assay. The values given represent the averages of assays on the sciatic nerves from each of three different normal and three different shiverer mice. The RIA was carried out as described by Cohen<sup>25</sup> and detects both  $A_1$  and  $P_1$  basic proteins.

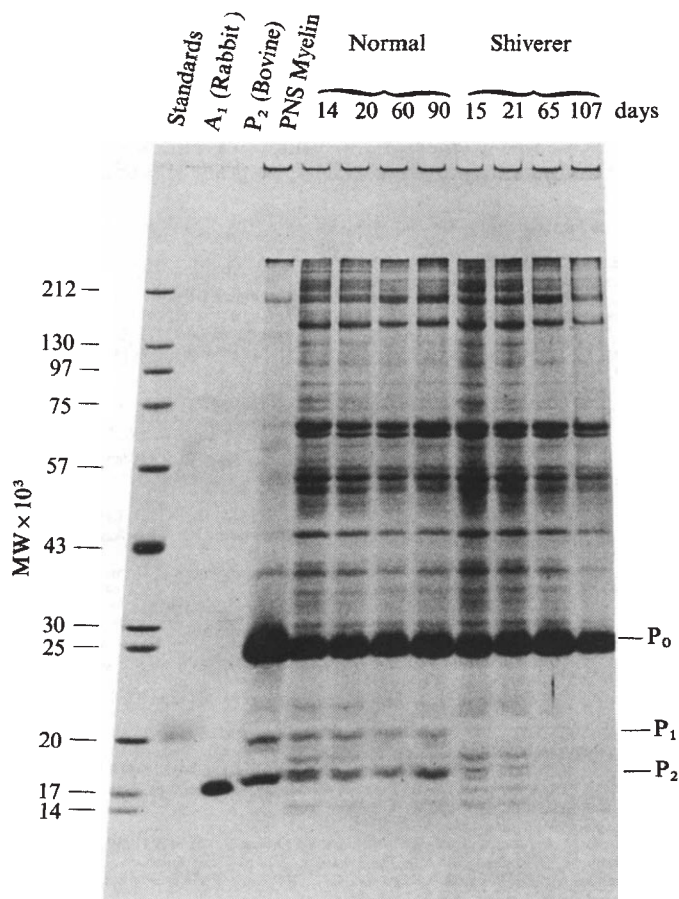


The proposed role of basic proteins as 'structural cement' in myelin compaction and stabilisation<sup>15-19</sup> derives from the physicochemical properties of basic protein from the CNS, where it accounts for 25-50% of the total protein of isolated myelin<sup>15</sup>. Because it is an extrinsic membrane protein, that is, easily extracted by dilute acid or salt solutions<sup>26</sup>, it is most probably localised to membrane surfaces. Large basic protein (named A<sub>1</sub> in CNS myelin) is highly cationic at physiological pH, with an isoelectric point of 10.5 (ref. 27) or 12-13 (ref. 28), and will complex with acidic lipids<sup>29</sup>, with which it forms lamellar systems<sup>30</sup>. CNS basic protein is also able to interact hydrophobically with lipids<sup>31</sup>, presumably due to the nonpolar regions in the protein molecule<sup>32</sup>. The basic proteins of PNS myelin have similar properties<sup>15,30,31,33,34</sup> and a similar structural role has therefore been suggested for them<sup>17</sup>. However, the lower amounts of basic proteins in PNS myelins, which range from 22% of total myelin protein in rabbit sciatic to as little as 6% in rat sciatic nerve<sup>35</sup>, lead one to question such a structural role for basic proteins in the PNS. Moreover, the virtual absence of basic proteins from shiverer peripheral nerve myelin demonstrates that these proteins are not required to maintain stable, compact myelin in the PNS. Possibly, the function of structural cement in peripheral nerve myelin is provided by P<sub>0</sub> glycoprotein, which constitutes 50-65% of the total myelin protein<sup>35</sup>. In contrast, if CNS myelin does require basic protein as a structural component, the absence of this protein as a result of the shiverer mutation<sup>7</sup> would explain the defective myelin formation of the CNS<sup>5,6</sup>. A preliminary account of this work has been reported elsewhere<sup>36</sup>.

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**Fig. 2** Electron micrographs from shiverer sciatic nerve. Upper: low magnification view ( $\times 2,400$ ) shows nerve fibres that seem to be normally myelinated. Lower: at high magnification ( $\times 434,000$ ) the major dense lines and the double intraperiod lines of the array of myelin membranes are apparent. Continuity of the intraperiod lines can be seen more clearly by viewing the micrograph obliquely. Nerves were fixed with glutaraldehyde, postfixed with ferrocyanide-OsO<sub>4</sub>, *en bloc* stained with uranyl acetate, dehydrated in methanol:methyl Cellosolve (1:1) and embedded in Epon; thin sections were counterstained with uranyl and lead.



**Fig. 3** SDS-PAGE analysis of protein composition of normal and shiverer sciatic nerves from animals of different ages. Nerve homogenates (15 mg wet weight per ml) were prepared in 2% SDS at room temperature, then diluted 1:1 with a mixture of 20 mM Tris-phosphate buffer at pH 6.7, 10%  $\beta$ -mercaptoethanol, 20% Ficoll and 0.002% bromophenol blue and finally stored frozen until electrophoresis. The electrophoretic system of Maizel<sup>20</sup> was used with the exceptions that the resolving gel was a slab 0.75 mm thick and 12 cm long, with an 8-18% gradient of acrylamide, and the stacking gel was 4% and made up in 62.5 mM Tris-phosphate at pH 6.7. Sample aliquots of 20  $\mu$ l per lane were electrophoresed at room temperature at a constant voltage of 40 V for 2 h, which brought the dye front into the resolving gel, and then 180 V for 4.5 h. The gels were fixed overnight in 50% methanol-10% acetic acid and subsequently stained for 1 h with 0.1% Coomassie Brilliant Blue R in 50% methanol-10% acetic acid. After destaining in 10% methanol-10% acetic acid the gels were photographed through a yellow filter (Kodak K 2). The molecular weight (MW) standards are: myosin at 212,000,  $\beta$ -galactosidase at 130,000, phosphorylase *a* at 97,000, conalbumin at 75,000, pyruvate kinase at 57,000, ovalbumin at 43,000, carbonic anhydrase at 30,000, chymotrypsinogen A at 25,000, soybean trypsin inhibitor at 20,000, myoglobin at 17,000, and lysozyme at 14,000. Myelin basic protein A<sub>1</sub>, prepared from rabbit brain<sup>39</sup>, is quite similar to P<sub>1</sub> basic protein of PNS<sup>33</sup>. Myelin basic protein P<sub>2</sub> was prepared from bovine intradural spinal roots<sup>40</sup>. PNS myelin was purified<sup>41</sup> from sciatic nerves of normal C57BL/6J mice. The major myelin proteins<sup>21</sup> of the sciatic nerve homogenates, P<sub>0</sub> glycoprotein and P<sub>1</sub> and P<sub>2</sub> basic proteins, are identified by comparison with the electrophoretic banding patterns of purified myelin basic proteins from other species and of myelin isolated from mouse sciatic nerves. P<sub>1</sub> and P<sub>2</sub> migrate in this gel system at the apparent MWs of ~20,000 and ~18,000; the actual MWs calculated from amino acid analysis of rabbit P<sub>1</sub> is 18,000 (ref. 21) and of bovine P<sub>2</sub> is 14,300 (ref. 42). Of the myelin proteins, P<sub>0</sub> glycoprotein is prominent in the gels of normal and shiverer mice, whereas P<sub>1</sub> and P<sub>2</sub> basic proteins are not visible in the shiverer. Two- and three-week-old normal and shiverer mice show more bands in the low MW region of the gels than do the adults. Two of these bands border the P<sub>2</sub> protein and are more apparent in shiverer because of the absence of this basic protein. They are, however, present in gels of normal nerves, especially at 14 days. Apart from the myelin proteins, the banding patterns at corresponding ages are virtually identical.

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## Evolution and gene transfer in purple photosynthetic bacteria

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A concern voiced in connection with recent sequencing of cytochrome *c* from the Rhodospirillaceae or purple non-sulphur photosynthetic bacteria<sup>1-3</sup> is that molecular information might be of little use in deciphering bacterial phylogeny because of the possibility of lateral transfer of genes and the consequent scrambling of the genetic record. This could be true for many proteins, of course, but the immediate question is, is it true for cytochromes *c*, for which extensive primary sequence<sup>1-3</sup> and X-ray structure<sup>4-10</sup> information is available for comparison? The evidence suggests that this is probably not so. The disagreement between cytochrome *c* sequences and the standard taxonomy of the Rhodospirillaceae in Bergey's Manual<sup>11</sup> is not a problem.

That reference, as its title indicates, is a manual of determinative rather than evolutionary bacteriology. Its goal is a reproducible system for identification of bacteria. If one claims that these determinative categories also have phylogenetic or evolutionary meaning, this is an assertion that must be proven. The argument outlined below suggests that molecular traits may eventually become more a dependable basis for classification of the Rhodospirillaceae than is gross morphology.

Table 1 contains information rearranged from Table 1 of Ambler *et al.*<sup>1</sup>, with the addition of two more species, and with further information about the positions of chain insertions in the cytochromes *c* and sequence comparisons between the 16S RNA subunits of bacterial ribosomes. The ribosomal RNA classification is discussed in greater detail in the following paper<sup>12</sup>, and more detailed information on cell shape and infolding of the photosynthetic membrane may be found in Pfennig and Trüper<sup>13</sup>. X-ray crystal structure analyses reveal that the cytochromes *c*<sub>2</sub> and *c*<sub>551</sub> from the Rhodospirillaceae fall naturally into three structural classes, each of which has a parallel among purely respiratory cytochromes *c*<sub>551</sub>, *c* or *c*<sub>550</sub>. These structural classes are shown in Fig. 1. The short (or S) cytochromes *c*<sub>551</sub> from *Rhodopseudomonas gelatinosa* and *Rhodospirillum tenue* resemble, in both sequence and folding, the respiratory *c*<sub>551</sub> from pseudomonads and *Azotobacter* (Fig. 1a). Cytochromes *c* of the medium or 'mitochondrial' (M) class are similar in length and sequence to cytochrome *c* from eukaryotic mitochondria, and all have the extra chain at the bottom of the molecule that is shaded and marked by I in Fig. 1b. Others, the long or L class, resemble *Paracoccus c*<sub>550</sub> by having the shaded insertions marked II and III in Fig. 1c, and sometimes insertion IV as well. Moreover, the inserted amino acids in these photosynthetic and respiratory cytochromes *c* are similar in sequence, arguing for a common evolutionary origin. All these molecules—*c*, *c*<sub>2</sub>, *c*<sub>550</sub>, *c*<sub>551</sub> and *c*<sub>555</sub> (from *Chlorobium*)—seem to make up one large evolutionarily related family of proteins. The presence or absence of chain insertions I, II, III and IV is listed as one of the traits in Table 1.

In Table 2, the amino acid sequences of cytochrome *c*<sub>2</sub> or *c*<sub>551</sub> from the Rhodospirillaceae are compared quantitatively in two different ways. Below the matrix diagonal, which contains the total number of amino acids in each protein, are tabulated the number of identical positions in pairwise comparisons of sequences. The number of differences between sequences is found above the diagonal. (As is customary, a deletion in one chain but not the other is included in the tally of differences.) For closely related sequences, the difference matrix is best for showing changes in sequence, but for more distantly related sequences such as these, the identities matrix is better. It avoids the difficult question of whether to treat a long chain deletion as one evolutionary event or many, and provides a measure of how nearly identical two sequences are in those regions of chain that they have in common. Gross insertions or deletions in the chain (such as regions I-IV) can then be assessed separately.

If the information in Tables 1 and 2 is arranged by Bergey genera, the principal impression is one of confusion. However, if the Bergey genera are set aside, a strong correlation can be seen between the size and sequence of cytochromes *c*, type of infolding of outer bacterial membrane, and 16S ribosomal RNA sequences. Those species that have class II RNA sequences also have short cytochromes *c* and tubular membrane infoldings, even though they fall into three different Bergey genera. (The amino acid sequence of *Rhodocyclus purpureus* cytochrome is not known, but one would predict that it is a short cytochrome.) Both the identity and the difference data in Table 2 show that the S cytochromes *c* are very different in sequences from the M and L (a mean and mean deviation of  $16.4 \pm 2.2$  identities between S and M or L). The M and L classes are more nearly alike, but there are fewer identities between M and L ( $38.6 \pm 3.3$  identities) than between different M ( $50.3 \pm 8.3$ ) or L ( $49.4 \pm 6.1$ ). The presence or absence of insertions II, III and IV, of course, draws a clear distinction between M and L cytochromes.



**Table 1** Comparison of properties of purple photobacteria and their cytochromes  $c_2$  or  $c_{551}$ 

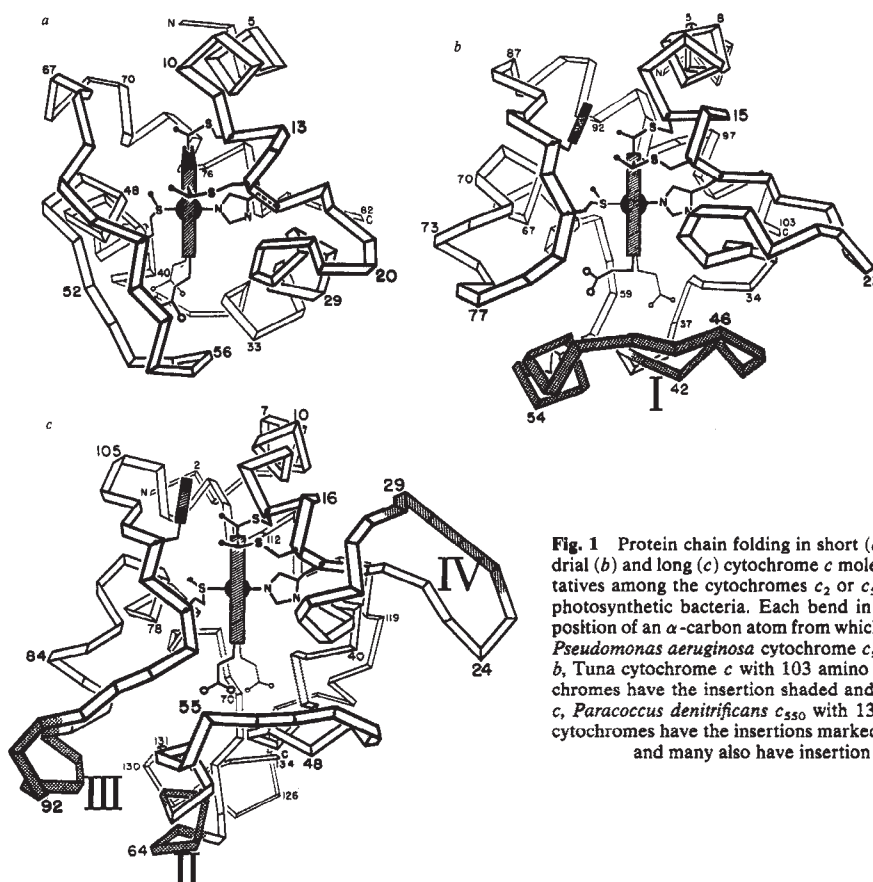
|                          | RNA Class | Cytochrome size | Chain insertions (Fig. 1) |     |       |     | Type of photosynthetic membrane | Mode of cell division | Cell form     |
|--------------------------|-----------|-----------------|---------------------------|-----|-------|-----|---------------------------------|-----------------------|---------------|
|                          |           |                 | I                         | II  | III   | IV  |                                 |                       |               |
| Chromatiaceae            | III       | ?               | ?                         | ?   | ?     | ?   | Mostly vesicular                | Fission               | Various       |
| Rhodospirillaceae        |           |                 |                           |     |       |     |                                 |                       |               |
| <i>Rps. gelatinosa</i>   | II        | S               | No                        | No  | No    | No  | Tubular                         | Fission               | Small rod     |
| <i>R. tenue</i>          | II        | S               | No                        | No  | No    | No  | Tubular                         | Fission               | Thin spiral   |
| <i>Rcy. purpureus</i>    | II        | ?               | ?                         | ?   | ?     | ?   | Tubular                         | Fission               | Arc or circle |
| <i>R. fulvum</i> *       |           | M               | Yes                       | No  | No    | No  | Lamellar                        | Fission               | Short spiral  |
| <i>R. molischianum</i> * |           | M               | Yes                       | No  | No    | No  | Lamellar                        | Fission               | Spiral        |
| <i>Rps. globiformis</i>  |           | M               | Yes                       | No  | No    | No  | Vesicular                       | Fission               | Sphere        |
| <i>Rm. vannielii</i>     | I         | M               | Yes                       | No  | No    | No  | Lamellar                        | Budding               | Stalked ovoid |
| <i>Rps. viridis</i>      | I         | M               | Yes                       | No  | No    | No  | Lamellar                        | Budding               | Rod           |
| <i>Rps. acidophila</i>   |           | M               | Yes                       | No  | No    | No  | Lamellar                        | Budding               | Large rod     |
| <i>Rps. palustris</i>    | I         | L               | Yes                       | Yes | Extra | No  | Lamellar                        | Budding               | Rod           |
| <i>R. photometricum</i>  |           | L               | Yes                       | Yes | Yes   | No  | Lamellar                        | Fission               | Large spiral  |
| <i>R. rubrum</i>         | I         | L               | Yes                       | Yes | Yes   | No  | Vesicular                       | Fission               | Spiral        |
| <i>Rps. capsulata</i>    | I         | L               | Yes                       | Yes | Yes   | Yes | Vesicular                       | Fission               | Rod           |
| <i>Rps. sphaeroides</i>  | I         | L               | Yes                       | Yes | Yes   | Yes | Vesicular                       | Fission               | Sphere        |

Abbreviations: *R.*, *Rhodospirillum*; *Rps.*, *Rhodopseudomonas*; *Rm.*, *Rhodomicrobium*; *Rcy.*, *Rhodocyclus*. S, M, L: short, medium and long cytochromes  $c$  as described in the text.

\* Possess two similar isozymes of  $c_2$ .

*Rps. gelatinosa*, *R. tenue* and *Rcy. purpureus* show a diversity of cell shapes, which led to their assignment to different genera in the original Bergey classification. If one accepts this separation, and argues that these three species show common cytochrome properties because of lateral gene transfer, one must not only assume that the genes for cytochromes  $c$  and for photosynthetic membrane configuration are closely linked and jointly transferrable, but that they are both also linked to the genes responsible for the 16S RNA components of their ribosomes. At this point, gross morphology begins to look like the weakest of the available traits for phylogenetic classification.

Those species with M or L cytochromes  $c$  all have class I ribosomal RNA sequences. Their cell membrane infoldings are vesicles or stacked lamellae, never tubules. The cells reproduce either by simple binary fission, or in four species by asymmetric budding. The limited information in Tables 1 and 2 does not provide a clear basis for subgroupings. One should keep an open mind on the subject until more information is available, perhaps from sequences and structures of proteins that are quite different from the cytochromes. However, Table 2 does indicate close affinities between *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata*, between *Rhodospirillum rubrum*



**Fig. 1** Protein chain folding in short (a), medium or mitochondrial (b) and long (c) cytochrome  $c$  molecules. All have representatives among the cytochromes  $c_2$  or  $c_{551}$  of purple non-sulphur photosynthetic bacteria. Each bend in the ribbon indicates the position of an  $\alpha$ -carbon atom from which a side chain branches. a, *Pseudomonas aeruginosa* cytochrome  $c_{551}$  with 82 amino acids<sup>10</sup>. b, Tuna cytochrome  $c$  with 103 amino acids<sup>8</sup>. All medium cytochromes have the insertion shaded and marked I at the bottom. c, *Paracoccus denitrificans*  $c_{550}$  with 134 amino acids<sup>6</sup>. All long cytochromes have the insertions marked II and III at the bottom, and many also have insertion IV at the right.

**Table 2** Pairwise sequence comparisons of cytochromes *c*

Italic numbers on the diagonal give the length of each chain. Below diagonal: total number of identical positions. Above diagonal: total number of differences. Sequences lettered at top as at far left.

|   |                          | Cytochrome size |    |    |   | D  | E   | F   | G   | H   | I   | J   | K   | L   | M   | N   |
|---|--------------------------|-----------------|----|----|---|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A | <i>Rps. gelatinosa</i>   | S               | 85 | 65 | — | 89 | 89  | 100 | 96  | 98  | 96  | 108 | 104 | 102 | 109 | 117 |
| B | <i>R. tenue</i>          | S               | 30 | 92 | — | 99 | 101 | 103 | 100 | 102 | 97  | 105 | 99  | 95  | 107 | 114 |
| C | <i>Rcy. purpureus</i>    | ?               | —  | —  | — | —  | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   |
| D | <i>R. fulvum</i> *       | M               | 18 | 16 | — | 99 | 12  | 71  | 58  | 65  | 66  | 82  | 77  | 77  | 85  | 94  |
| E | <i>R. molischianum</i> * | M               | 18 | 14 | — | 88 | 100 | 68  | 56  | 66  | 64  | 80  | 74  | 78  | 86  | 94  |
| F | <i>Rps. globiformis</i>  | M               | 12 | 16 | — | 37 | 40  | 106 | 62  | 66  | 65  | 83  | 81  | 82  | 92  | 97  |
| G | <i>Rm. vannielii</i>     | M               | 15 | 16 | — | 48 | 50  | 47  | 104 | 52  | 48  | 73  | 76  | 74  | 85  | 89  |
| H | <i>Rps. viridis</i>      | M               | 16 | 14 | — | 44 | 43  | 47  | 56  | 107 | 47  | 76  | 79  | 79  | 84  | 91  |
| I | <i>Rps. acidophila</i>   | M               | 18 | 19 | — | 43 | 45  | 47  | 59  | 61  | 107 | 80  | 80  | 76  | 90  | 81  |
| J | <i>Rps. palustris</i>    | L               | 13 | 18 | — | 37 | 39  | 40  | 48  | 48  | 44  | 114 | 74  | 72  | 72  | 88  |
| K | <i>R. photometricum</i>  | L               | 14 | 21 | — | 39 | 42  | 37  | 40  | 41  | 39  | 45  | 113 | 50  | 79  | 82  |
| L | <i>R. rubrum</i>         | L               | 16 | 25 | — | 38 | 37  | 36  | 41  | 40  | 42  | 46  | 63  | 112 | 68  | 75  |
| M | <i>Rps. capsulata</i>    | L               | 15 | 18 | — | 36 | 35  | 33  | 36  | 40  | 34  | 52  | 41  | 51  | 116 | 62  |
| N | <i>Rps. sphaeroides</i>  | L               | 13 | 15 | — | 33 | 33  | 32  | 36  | 37  | 46  | 42  | 43  | 49  | 62  | 124 |

\* Iso-2 is similar.

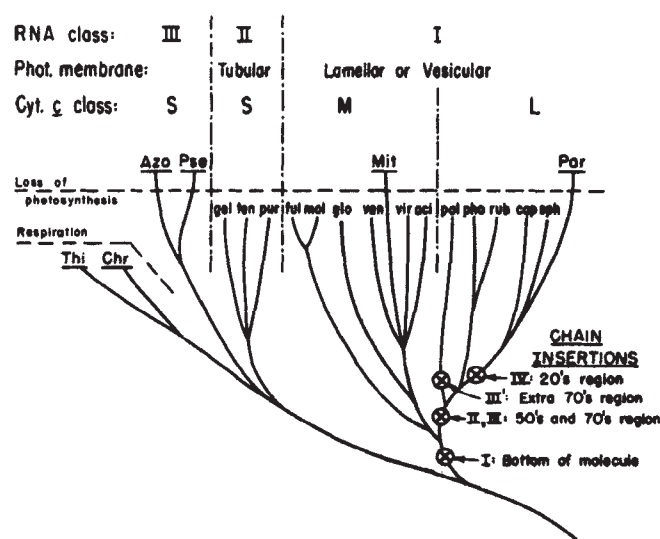
and *Rhodospirillum photometricum*, between three and possibly the fourth budding species, and between *Rhodospirillum molischianum* and *Rhodospirillum fulvum*, all of which are reasonable on other grounds. We have very tentatively drawn the phylogenetic tree shown in Fig. 2 as the basis for future discussion of new data. It suggests a point that we believe to be quite important, and which was first enunciated by Broda<sup>14,15</sup>: the polyphyletic origin of respiring bacteria from more than one line of photosynthetic ancestors.

It may be that lateral gene transfer and blurring of the evolutionary record will be a serious problem for some proteins, especially those that are intrinsically useful to a bacterium in their own right, without an attendant metabolic setting. However, with the proper choice of proteins this difficulty should be capable of being overcome, and the cytochrome *c* record provides grounds for optimism.

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**Fig. 2** Tentative phylogenetic tree of the purple photobacteria, based on the metabolic, morphological, protein and RNA data presented in Tables 1 and 2. The original cytochrome *c* is assumed to have been small, like *Chlorobium* *c*<sub>555</sub> and *Anacystis* *c*<sub>554</sub>. Circled crosses mark the four places where specific insertions of polypeptide chain are presumed to have occurred. Azo, *Azotobacter*; Pse, *Pseudomonas*; Mit, eukaryotic mitochondria; Par, *Paracoccus denitrificans*; Thi, *Thiobacillus*; Chr, *Chromatium*; gel, *Rps. gelatinosa*; ten, *R. tenue*; pur, *Rhodocyclus purpureus*; ful, *R. fulvum*; mol, *R. molischianum*; glo, *Rps. globiformis*; van, *Rm. vannielii*; vir, *Rps. viridis*; aci, *Rps. acidophila*; pal, *Rps. palustris*; pho, *R. photometricum*; rub, *R. rubrum*; cap, *Rps. capsulata*; sph, *Rps. sphaeroides*.

## Do genealogical patterns in purple photosynthetic bacteria reflect interspecific gene transfer?

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It is generally thought that interspecific (lateral) transfer of genes is so extensive among bacteria that it is difficult, and perhaps impossible, to determine their phylogenetic relationships. Ambler and coworkers<sup>1</sup> reflect this in their suggestion that the relationships seen among cytochrome *c* sequences of the *Rhodospirillaceae* are merely the result of a haphazard lateral transfer of the particular gene, and give no indication of the true bacterial phylogenies. However, if comparative analysis of several unrelated macromolecules yields essentially the same phylogenetic tree, then that pattern is extremely unlikely to reflect the lateral transfer of genes<sup>2</sup>. We have also determined 16S ribosomal RNA catalogues<sup>3</sup> for many of the *Rhodospirillaceae* investigated by Ambler *et al.* and here we use these two sets of data to compare molecular phylogenies for these bacteria.



The conclusion of Ambler *et al.*<sup>1</sup>, suggesting that the relationships seen among cytochrome *c* sequences of the Rhodospirillaceae are merely due to random lateral transfer of the relevant gene, is based on three arguments. First, within the purple photosynthetic bacteria there is 'no correlation between a species' cytochrome *c* amino acid sequence and its phylogenetic position'; second, the cytochromes *c* from some of the Rhodospirillaceae seem to be more closely related to those from certain nonphotosynthetic organisms, for example pseudomonads, than they are to those from other members of the Rhodospirillaceae; and third, three different proteins—cytochrome *c*<sub>551</sub>, cytochrome *c*', and the high potential iron sulphur protein—seem to produce three dissimilar phylogenetic trees for the same (small) set of organisms<sup>1,4</sup>. The first two arguments assume that we already know the true phylogeny for the Rhodospirillaceae, which is not the case. The genera now defined for the purple photosynthetic bacteria are not intended to be phylogenetic categories<sup>5,6</sup>; generally agreed phylogenetic categories for these organisms have never been proposed. In addition, the second argument seems to reflect the idea that the photosynthetic bacteria must be phylogenetically quite distinct from nonphotosynthetic bacteria (with perhaps a few exceptions). Although this view is widely accepted<sup>7,8</sup>, it has no factual support.

Our objection to the lateral transfer explanation is not based primarily on counter-argument, however, but on the facts available. If a phylogeny derived from a comparative analysis of one macromolecular species reflects lateral transfer of the underlying gene, then it is virtually impossible that the same phylogeny will result when another functionally unrelated macromolecule is used as the phylogenetic probe<sup>2</sup>.

Many of the Rhodospirillaceae whose cytochromes *c* have been sequenced by Ambler *et al.*<sup>1</sup> have also been characterised in terms of their 16S ribosomal RNAs using the oligonucleotide cataloguing method<sup>3</sup>. Both data sets are analysed by comparable methods: the 16S rRNA catalogues are compared by the binary association coefficient *S*<sub>AB</sub> as described previously<sup>9</sup>; and the cytochrome *c* data are represented as per cent sequence homology for each pair of sequences (using the alignments of Dickerson<sup>10</sup>, see Table 2 of the accompanying paper<sup>10</sup>). Cluster analysis (average linkage between the merged groups) on these two data sets generates the dendrograms shown in Fig. 1.

The two patterns are in excellent agreement. It is therefore highly unlikely that either one reflects the lateral transfer of genes. Moreover, a number of phenotypic properties of these organisms are consistent with the groups so defined (for details see refs 3, 6 and 10).

The 16S rRNA data indicate that the purple photosynthetic bacteria fall into three major classes—that defined by the bulk of the *Rhodospseudomonas* species and their relatives (class I), that defined by *Rhodospseudomonas gelatinosa* and *Rhodospirillum tenue* (class II) and that defined by *Chromatium* (class III). The first two classes, which comprise the purple non-sulphur bacteria, give no evidence of being specifically related to one another to the exclusion of the last class, the purple sulphur bacteria. Thus if, as has been suggested<sup>10</sup>, the former phenotype evolved from the latter, it may have done so several times.

Although a comprehensive survey of 16S rRNA catalogues for the Gram-negative bacteria is not yet available, it is already apparent that the genealogical cluster defined by the purple photosynthetic bacteria is far more extensive phylogenetically than had been generally realised; each of the above-defined three classes of purple photosynthetic bacteria also contains representatives of various major nonphotosynthetic Gram-negative groups, for example, pseudomonads, enterics and vibrios<sup>8</sup>. Thus it would seem that many of the Gram-negative bacteria have arisen as nonphotosynthetic offshoots of photosynthetic ancestors, and the theory that the photosynthetic groups of bacteria are largely phylogenetically distinct from nonphotosynthetic groups is incorrect.

An alternative explanation for the third argument of Ambler *et al.*—the non-congruence of phylogenetic trees derived from

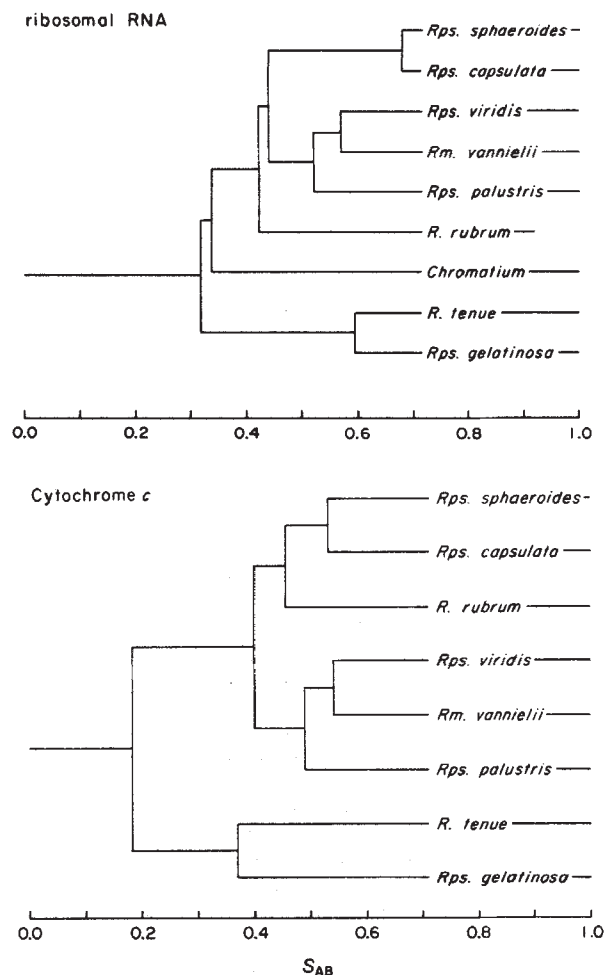


Fig. 1 The relationship among various purple photosynthetic bacteria as determined by 16S ribosomal RNA sequence comparisons, and cytochrome *c* sequence comparisons. *Rps.*, *Rhodospseudomonas*; *R.*, *Rhodospirillum*; *Rm.*, *Rhodomicrobium*.

different proteins—is that their finding reflects the limitations in the use of small macromolecules such as cytochrome *c* for genealogical measurement. For a macromolecule to be useful as an evolutionary clock it must maintain functional and tertiary structural constancy over the group of organisms being considered<sup>11,12</sup>. Changing these collective properties seemingly involves clusters of selected, and therefore relatively rapid, changes in genetic sequence, making the molecule an inexact measure of phylogenetic distance at best. Bacterial genealogies encompass considerably greater phylogenetic distances and varieties of niches than genealogies within the higher organisms. Consequently it is not surprising that the conditions of constancy of tertiary structure and function are often not found in the bacteria. The three types of protein studies by Ambler *et al.*—cytochromes *c*<sub>2</sub> and *c*<sub>551</sub>, cytochrome *c*' and the high potential iron sulphur protein<sup>4</sup>—seem to confirm this point. It is, therefore, not surprising that these three types of macromolecules yield non-congruent phylogenetic trees. For reasons not discussed here we feel that a large macromolecule such as the 16S rRNA is less susceptible to the difficulties which tend to arise when using the smaller macromolecules as genealogical probes.

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## Isolation of the structural gene for alcohol dehydrogenase by genetic complementation in yeast

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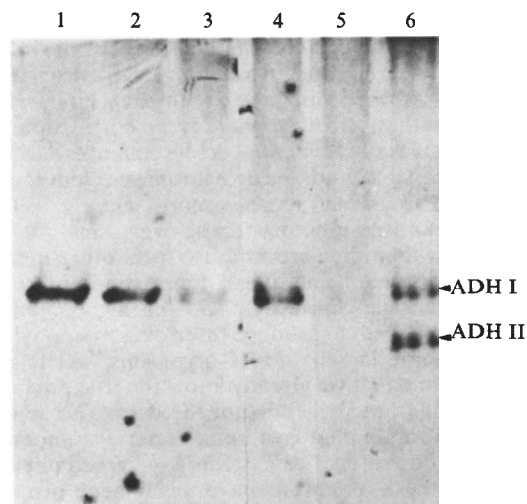
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**Alcohol dehydrogenase (ADH) catalyses the interconversion of acetaldehyde and ethanol. This reaction is the final step during alcoholic fermentation in yeast and the initial step in the metabolism of ethanol in a wide variety of organisms. The regulation of ADH is now being studied in several organisms, notably yeast<sup>1-4</sup>, *Drosophila*<sup>5</sup>, maize<sup>6</sup> and man<sup>7,8</sup>. These organisms contain multiple ADH isozymes which are differentially expressed. The budding yeast *Saccharomyces cerevisiae* is particularly suitable for study because of the potential for genetic analysis of enzyme regulation and structure. Yeast possesses three distinct ADH isozymes<sup>2</sup>. In the presence of glucose ADHI (the 'classical' yeast ADH) is expressed whereas ADHII is repressed. A third enzyme, mADH, is associated with mitochondria. Mutants lacking each of these enzymes have been isolated by virtue of their greater tolerance to allyl alcohol<sup>1</sup>, which is converted by ADH to the toxin, acrolein. There is no evidence as to whether the regulation of any one of these enzymes is under transcriptional control. Availability of recombinant DNA clones containing the respective genes would be of obvious value in resolving this problem. We describe here the use of the newly developed technique of yeast transformation<sup>9-11</sup> to isolate recombinant plasmids that contain the structural gene for ADHI (*ADC1*). These plasmids were selected from a pool of plasmids containing yeast DNA by virtue of their ability to complement the growth defect of yeast strains completely lacking ADH activity. Using DNA sequencing, they have been shown to contain the structural gene for ADHI.**

The vector used for constructing the yeast DNA pool was the plasmid YRp7 (ref. 11). YRp7 is composed of a 1.4-kilobase *EcoRI* fragment containing the yeast *TRP1* gene inserted into the *EcoRI* site of pBR322 (ref. 12). The fragment containing *TRP1* confers on the hybrid plasmid the ability to replicate autonomously in yeast as a low copy number closed circular molecule<sup>11</sup>. Because it need not integrate into the chromosome, YRp7 will transform *trp1* strains with high efficiency ( $10^4$ - $10^5$  transformants per  $\mu$ g DNA).

The construction of a pool of yeast sequences in YRp7 will be described in more detail elsewhere (K. N. and S. I. Reed, in preparation). Briefly, yeast DNA was partially cleaved with restriction endonuclease *Sau3A*, a 4-base pair recognition site enzyme which produces cohesive ends homologous to those produced by *BamHI*. The DNA fragments were separated by centrifugation in sucrose gradients and fragments larger than 5 kilobases were ligated into the *BamHI* site in the tetracycline-resistance gene of YRp7. The pool of recombinant plasmids so



**Fig. 1** Screening of transformed yeast for ADH activity. A *trp1* strain lacking all ADH isozymes (302-21; *mata*, *trp1*, *his4*, *adc1*, *adm*, *adr2*) was selected from a cross of strain SF-1C-M3 (donated by M. Ciriacy) and strain H293-4-2 (provided by L. Hartwell). The procedure for yeast transformation was essentially that described by Beggs<sup>10</sup>. Cells were plated in a 3% agar overlay lacking tryptophan and containing 2% glucose. The nine large *Trp*<sup>+</sup> colonies obtained after transformation with the YRp7 yeast pool were plated on medium lacking tryptophan. Extracts from cells grown to stationary phase in medium lacking tryptophan were analysed for ADH activity by spectrophotometric analysis of *NAD*<sup>+</sup> reduction when ethanol was added as substrate<sup>1</sup>. Aliquots of cell extracts containing approximately 22  $\mu$ g of protein were electrophoresed into a 5% polyacrylamide slab gel. The electrophoresis system used was adapted from the Tris-glycine system of Davis<sup>18</sup>. The sample was applied to the gel in buffer containing 50 mM Tris-HCl, pH 6.8, 25% glycerol and 100 mM  $\beta$ -mercaptoethanol, with bromophenol blue as an indicator. The stacking gel consisted of 3% acrylamide, 0.08% bisacrylamide, 0.12% TEMED, 0.2% ammonium persulphate and 125 mM Tris-HCl, pH 6.8. The running gel consisted of 5% acrylamide, 0.13% bisacrylamide, 0.03% TEMED, 0.3% ammonium persulphate and 375 mM Tris-HCl, pH 8.8. Electrophoresis buffer was 25 mM Tris and 192 mM glycine and electrophoresis was carried out at 150 V for 3 h at 4°C. The gel was then stained for ADH activity<sup>19</sup>. In the example shown, wells 1-5 contain extracts from the transformants 9T1-9T5, respectively. Well 6 contains extract from the strain which was used as the yeast DNA source in constructing the DNA vector pool.

created (>25,000 independent *tet*<sup>r</sup> clones) was stored and amplified by transformation of *Escherichia coli* to ampicillin resistance.

The selective technique used for identifying plasmids containing an ADH gene was based on the inability of yeast mutants lacking ADHI to grow in the absence of respiration<sup>13</sup>. Such mutants will not grow at all in the presence of antimycin A (M. Ciriacy, personal communication) and grow only poorly when embedded in a 3% agar overlay. A *trp1* strain lacking all ADH isozymes was transformed with plasmid DNA prepared from the pool using the technique described by Beggs<sup>10</sup> (see Fig. 1 legend). Nine large colonies (9T1-9T6; 10T1-10T3), a frequency of one per  $4 \times 10^3$  *Trp*<sup>+</sup> transformants, were produced after incubation at 30°C for 1 week. Seven out of the nine contained ADH activity after growth to stationary phase (Fig. 1). When analysed by electrophoresis on a native polyacrylamide gel, all seven displayed a band of activity co-migrating with ADHI from the donor strain (Fig. 1).

The *Adc*<sup>+</sup> transformants fell into two classes. Two, 9T2 and 10T2, had ADHI levels comparable to wild type. They were allyl alcohol sensitive, antimycin A resistant and stable for the *Trp*<sup>+</sup> phenotype. These stable transformants were further analysed by mating with an ADH-null *trp1* strain. Tetrad analysis of the haploid progeny showed that both the *Trp*<sup>+</sup> and

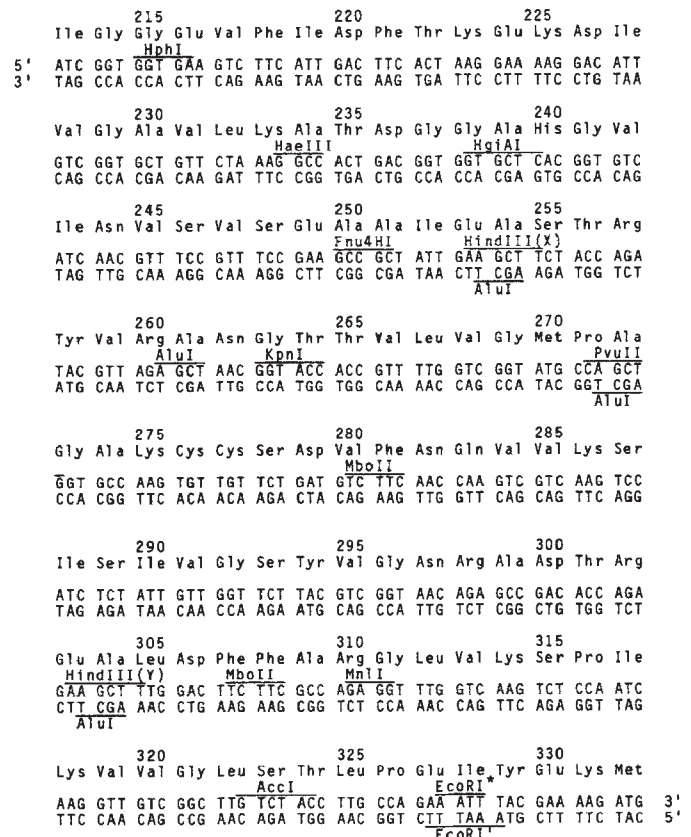


*Adc*<sup>+</sup> phenotypes segregated 2:2 (*Trp*<sup>+</sup>*Adc*<sup>+</sup>:*Trp*<sup>-</sup>*Adc*<sup>-</sup>), that is, only parental combinations were observed. This result indicates that these normally unlinked genes are now linked and have integrated into a chromosomal site. The site of integration was not determined. The second class of transformants contained 10–100% of the wild-type ADHI activity. When plated on YEPD plates, cells from these transformants produced a mixture of large and small colonies and rapidly lost the ability to grow without tryptophan. These colonies presumably contained autonomously replicating plasmids which were readily lost in the absence of selection, a property characteristic of YRp7 (ref. 11).

Plasmid DNA was isolated from transformant 9T6 and amplified in *E. coli* as described in Fig. 2 legend. Restriction enzyme digestion of the plasmid DNA (pY9T6) isolated from three different *E. coli* clones transformed with plasmid DNA from transformant 9T6 showed that all were identical. Three different plasmids, one of which (pY9T5) shared common yeast restriction fragments with pY9T6, were similarly isolated from yeast transformant 9T5.

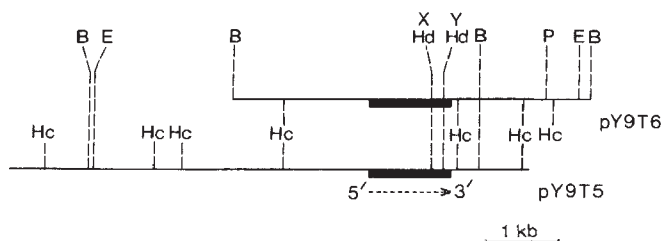
Plasmids pY9T5 and pY9T6 were used to retransform strain 302–21 (*ADH*-null). With pY9T6, 0.06% of the cells surviving treatment were transformed to *Trp*<sup>+</sup>, and with pY9T5, 0.02% were similarly transformed. All *Trp*<sup>+</sup> cells were able to grow in the presence of antimycin A. Eight transformants were grown in medium lacking tryptophan and the extracts were assayed for ADH activity. All had near wild-type levels of ADH activity and produced an active enzyme which co-migrated with ADHI during electrophoresis. Both the *Trp*<sup>+</sup> and *Adc*<sup>+</sup> phenotypes were unstable in the absence of selection and were lost coordinately.

The above results clearly show that both pY9T5 and pY9T6 are capable of complementing the *ADHI* deficiency of 302–21



**Fig. 3** Partial DNA sequence of the *ADC1* locus. The DNA sequence coding for amino acid residues 213–332 in the ADHI protein was determined by the technique of Maxam and Gilbert<sup>23</sup>. 100 µg of pY9T6 DNA was limit digested with *Hind*III then dephosphorylated with bacterial alkaline phosphatase and labelled with <sup>32</sup>P at the 5' end using T4 polynucleotide kinase as described by Richardson<sup>24</sup>. DNA was then precipitated with ethanol to remove unreacted [<sup>32</sup>P]ATP. Half the labelled DNA was base denatured and loaded onto a long (38 cm) 6% native acrylamide gel. Electrophoresis for 18 h at 150 V resolved the separated strands of the 150-base pair restriction fragment between *Hind*III sites X and Y (Fig. 2). DNA fragments containing the other two pertinent labelled ends were isolated on 0.7% agarose gels, electroeluted, purified over DE-52, cleaved with a second restriction enzyme and resolved on another 0.7% agarose gel. Modifications and cleavages of the four electroeluted DNA samples were done by standard Maxam and Gilbert procedures<sup>23</sup>. Products were analysed on 8% and 20% acrylamide, 7 M urea sequencing gels which were either 0.5 mm or 0.7 mm thick. All but one of the 120 amino acids derived from the DNA sequence agree with the published amino acid sequence of the ADHI protein<sup>14,15</sup> for amino acid residues 213–332. Our sequence demonstrates that residue 236 is aspartic acid rather than asparagine. The restriction endonuclease sites shown were detected by scanning the derived DNA sequence. Only the *Hae*III and *Hind*III sites have been confirmed by actual restriction endonuclease digestion.

caused by the *adc1* mutation. However, apart from the fact that ADHI-defective mutants have been found only at this locus<sup>2</sup>, there is little compelling evidence that the *ADC1* locus in fact constitutes the structural gene for the enzyme. To resolve this problem, we have tested the ability of pY9T5 and pY9T6 to hybridise to a <sup>32</sup>p-labelled cDNA probe prepared from an RNA fraction highly enriched for ADHI mRNA (J.B., unpublished). Both plasmids hybridised strongly (results not shown), suggesting that the locus is indeed the structural gene. Restriction maps of pY9T5 and pY9T6 (Fig. 2) show that the two plasmids contain about 4.1 kilobases of overlapping sequence but that their inserts are of different sizes and are in the opposite orientation in the YRp7 vector. DNA sequence was obtained



**Fig. 2** Restriction endonuclease cleavage sites on the overlapping yeast DNA inserts of recombinant plasmids pY9T5 and pY9T6. Plasmid DNA was prepared as described by Livingston and Klein<sup>20</sup> from the unstable yeast transformants after growth in the absence of tryptophan and in the presence of antimycin A. Transformation of *E. coli* to ampicillin resistance produced approximately 100 colonies per µg DNA, all of which were tetracycline sensitive. Plasmid DNA was purified from *E. coli* by a modification of the procedure of Elwell *et al.*<sup>21</sup>. Single and double restriction digests of the DNA were analysed on horizontal 0.7% agarose gels. The position of the *ADC1* locus was first determined by hybridisation to Southern filter<sup>22</sup> replicas of these agarose gels with radioactive cDNA complementary to an ADHI mRNA-enriched RNA fraction. A detailed description of the preparation and use of this cDNA will be presented elsewhere. The position and extent of the *ADC1* structural locus was further defined by the DNA sequencing results (Fig. 3). The cleavage sites of relevant restriction nucleases are designated as follows: B(*Bam*HI), E(*Eco*RI), Hc(*Hinc*II), Hd(*Hind*III), P(*Pst*I); X and Y designate specific *Hind*III sites which are referred to in the text and Fig. 3. The extent and position of the structural *ADC1* locus is shown by the solid bars and the direction of transcription by the arrow below the bars. Restriction maps of the entire plasmids (not shown) demonstrated that the *ADC1* locus is in the opposite orientation in pY9T5 and pY9T6 relative to the YRp7 vector sequences. The *Bam*HI site at the left end of pY9T6 is a cloning artefact produced by ligation of the *Sau*3A sticky end into the pBR322 *Bam*HI site. The pY9T5 insert is 7.2 kilobases and the pY9T6 insert 4.9 kilobases. Overlap between pY9T5 and pY9T6 defines a 4.1-kilobase sequence which is sufficient for ADHI expression.

for the region surrounding *Hind*III sites X and Y (Fig. 3). Comparison of this sequence with the known amino acid sequence for ADHI<sup>14</sup> shows conclusively that these plasmids contain the structural gene. Figure 3 shows that the DNA sequence corresponds to an uninterrupted sequence encoding amino acids 213–332 in the ADHI protein with only one amino acid difference from the published sequence<sup>14,15</sup>. The extent of the coding sequence, if there are no intervening sequences, is indicated in Fig. 3. The ability of these plasmids to complement the growth defects of an ADH-null strain, together with the presence of normal levels of ADH activity, demonstrates that a functioning ADHI gene is present on both pY9T5 and pY9T6.

One reason for investigating ADH in yeast is to compare two genes which code for closely related proteins, ADHI and ADHII<sup>15</sup>, but which are expressed in different conditions. As mentioned earlier, all the transformants which were selected for ADH activity contained ADHI, the constitutive enzyme. The absence of transformants containing ADHII was to be expected because the repression of this enzyme by the glucose present in our conditions of transformation would prevent it from complementing the growth defect of the recipient strain. The use of DNA from an available constitutive mutant of ADHII<sup>4</sup> should alleviate this problem and allow the ADHII structural gene (*ADR2*) to be cloned in a similar manner to *ADC1*. Alternatively, as ADHI and ADHII differ in only 15 of 284 amino acid positions<sup>15</sup>, it is likely that their DNA sequences will cross-hybridise sufficiently to allow the already cloned *ADC1* sequence to be used as a hybridisation probe for *ADR2*.

The use of complementation to isolate genes from a plasmid or phage pool is not new. Several yeast genes, for instance, *LEU2*, *HIS3*, *URA3*, and *TRP1*, have been isolated<sup>11,16,17</sup> by virtue of their ability to complement equivalent mutations in *E. coli*. By using yeast rather than *E. coli* cells as the recipient in transformation experiments, this same approach can be used to clone genes for which there is no analogous *E. coli* function. Preliminary experiments in our laboratory have shown that this potential is realisable for several different types of gene, for instance, those necessary for the cell division cycle (K. N. Nasmyth and S. I. Reed, in preparation) and mating. One might also speculate, based on the precedent set by the expression of yeast genes in *E. coli*, that yeast transformation may be a valuable technique for identifying and isolating genes from other eukaryotes. In particular, the system described here may prove useful for the isolation of ADH genes from other organisms.

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## Trans-complementable copy-number mutants of plasmid ColE1

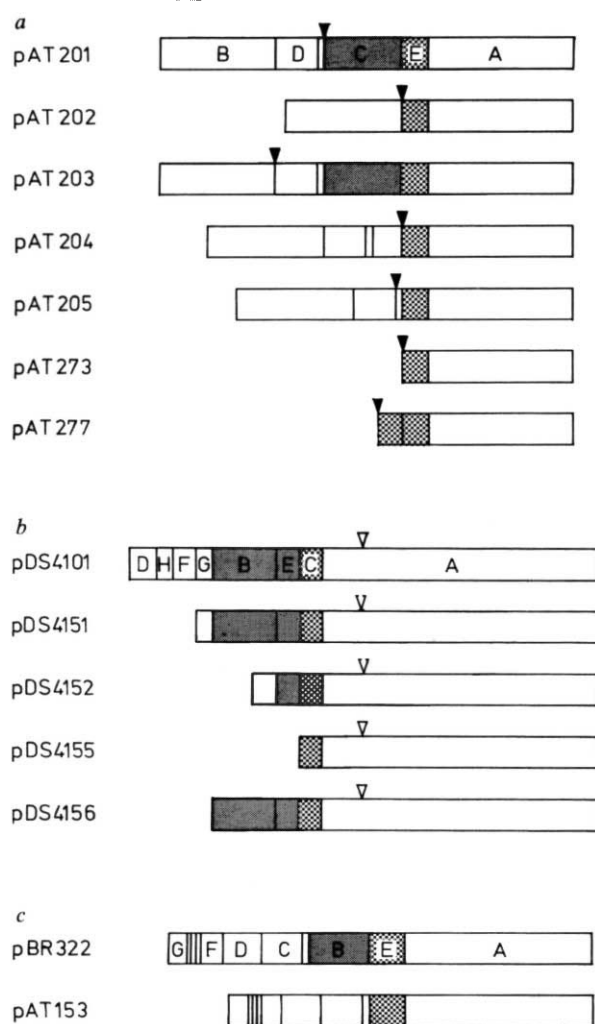
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Plasmid ColE1, like many other small non-conjugative plasmids, is present in multiple copies (about 15 per chromosome equivalent) in *Escherichia coli* cells<sup>1,2</sup>. Because of their high copy number, the replication of such plasmids has been described as 'relaxed', even though there is good evidence that it is strictly controlled: ColE1 derivatives have characteristic but different copy numbers<sup>2</sup> and ColE1 copy-number mutants have been characterised<sup>3–5</sup>. No plasmid-specified protein is essential for the replication of ColE1 and related plasmids, as extensive replication can occur in chloramphenicol-treated cells<sup>6</sup>, in plasmid-free chloramphenicol-treated cells transfected with a hybrid ColE1/phage replicon and *in vitro* in extracts derived from plasmid-free cells<sup>8</sup>. Nevertheless, it is possible that a plasmid-specified protein is involved in ColE1 replication control in viable cells. Here we show that deletion of a given non-essential region from ColE1-like plasmids results in a raised copy number. Such plasmids are stably maintained and have their copy number returned to normal when a complementing plasmid is present in the same cell, indicating that a plasmid-specified diffusible gene product regulates the plasmid content of ColE1-containing cells. Deletion of the equivalent region from the cloning vector pBR322 gives a derivative which has a raised copy number and which has also lost its origin for conjugal transfer; unlike pBR322, it cannot be mobilised.

Several chloramphenicol-resistant (Cm<sup>r</sup>) deletion derivatives of ColE1 were constructed by mixing partially *Hae*II-digested ColE1 DNA with a purified *Hae*II fragment, specifying Cm<sup>r</sup>, of pACYC184 (ref. 9). After ligation *in vitro*, the DNA was transformed into *E. coli* K12 [DS410 (ref. 10)], selecting for Cm<sup>r</sup>. The structure of some of the resulting plasmids is given in Fig. 1(a) and the plasmid DNA content of cells containing these mutants is shown in Table 1. For all mutants tested (including others not shown here), there is a direct correlation between raised plasmid level and the absence of the ColE1/*Hae*II-C fragment. Mutants containing *Hae*II-C have a plasmid level close to that of ColE1, whereas those deleted for *Hae*II-C have a five to sevenfold increase in plasmid content. To determine whether *Hae*II-C contains all or part of a gene specifying a repressor for copy-number control and/or a site at which it acts, we attempted the complementation of raised copy number using the related plasmid, ColK. Although this plasmid is compatible with ColE1<sup>11</sup>, the two plasmids show partial homology (D.S., unpublished) and have interchangeable mobility functions<sup>11</sup>. The effect of ColK and some of its deletion derivatives (Fig. 1b) on the ColE1 copy mutants is shown in Table 1. Whereas the presence of ColK reduced the plasmid level of all ColE1 copy mutants, the unrelated plasmid pSC101 and some of the ColK derivatives had little effect on ColE1 plasmid content. This suggests that we are observing true complementation rather than a nonspecific lowering of individual plasmid content. Examination of the structure of the ColK plasmids that have no effect on the ColE1 copy mutants indicates that the complementing gene product (repressor) is specified by a gene whose expression to give an active gene product disappears when ColK/*Hae*II-B is deleted; the gene could be wholly contained on the fragment or extend into the E or even C fragment of ColK. This region corresponds in position and is probably partially homologous to the *Hae*II-C fragment of ColE1, thus supporting the idea that this region in both ColE1 and ColK encodes a repressor of plasmid replication. The site of action of this repressor must be present in all the copy mutants.





**Fig. 1** Structures of the plasmids used. *a*, Cm<sup>r</sup> ColE1 derivatives. pAT201, and pAT203 have the entire ColE1 genome with the Cm<sup>r</sup> *HaeII* fragment of pACYC184<sup>9</sup> (▼) inserted at either of two *HaeII* sites; the other plasmids have various ColE1 *HaeII* fragments deleted. *b*, Ap<sup>r</sup> ColK derivatives. pDS4101 is ColK::Tn1. The other plasmids are deletion derivatives of this. ▼ Indicates intact Tn1, and V indicates that some of the Tn1 internal *HaeII* fragments have been deleted. *c*, pBR322 and its *HaeII* deletion derivative pAT153. Vertical lines indicate *HaeII* sites. Light stippling indicates the *HaeII* fragment carrying the origin of replication and heavy stippling indicates the region encoding the gene product (repressor) that maintains normal plasmid copy number.

**Table 2** Conjugal mobility of plasmid cloning vectors

| Plasmid vector | Mobilisation promoted by conjugative plasmid R64drd11* | Mobilisation from a R64 drd11 ColK <sup>+</sup> cell |
|----------------|--------------------------------------------------------|------------------------------------------------------|
| pBR322         | $<8 \times 10^{-6}$                                    | $9 \times 10^{-2}$                                   |
| pAT153         | $<1 \times 10^{-5}$                                    | $<4 \times 10^{-6}$                                  |
| pACYC184       | $<9 \times 10^{-6}$                                    | $<2 \times 10^{-5}$                                  |

\* Frequency of R64-receiving recipients that inherit given factor.

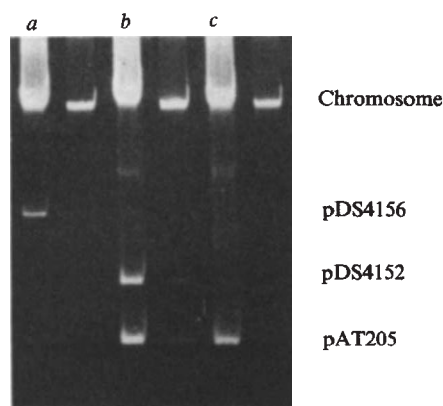
† ColK is a natural relative of ColE1 that specifies *trans*-acting mobility proteins that are interchangeable with those of ColE1 for R64drd11-promoted transfer<sup>11</sup>.

To test if deletion of the equivalent region from plasmids derived from natural relatives of ColE1, results in increased copy number, we partially digested with *HaeII* the cloning vector pBR322 (refs 12, 13), which is ampicillin resistant (Ap<sup>r</sup>) and tetracycline resistant (Tc<sup>r</sup>) and is derived from pMB1, closely related to ColE1<sup>14</sup>. Inspection of the published sequence of the pBR322 *HaeII*-B fragment<sup>13</sup> with that available to us for the ColE1 *HaeII*-C fragment, shows that most of pBR322 *HaeII*-B is contained within ColE1 *HaeII*-C in a highly conserved form (also see ref. 13, page 85). After *in vitro* ligation and transformation, selecting for Ap<sup>r</sup>Tc<sup>r</sup>, we isolated a deletion derivative lacking the 0.62-kilobase *HaeII*-B fragment of pBR322 (Fig. 1c). Cells containing this plasmid (pAT153) have a 1.5–3-fold greater plasmid content than those containing the parental plasmid. Although this increase is smaller than that for the ColE1 deletion derivatives, we believe that it makes pAT153 a potentially useful cloning vector because of greater plasmid and plasmid gene product yields per ml of culture. pAT153, like pBR322, replicates in chloramphenicol-treated cells. In terms of biological containment for *in vitro* genetic manipulation, pAT153 also has a great advantage over pBR322 in that it has lost *nic*, the transfer origin for conjugal transfer. Whereas pBR322 is efficiently mobilised from cells containing the appropriate conjugative plasmid and ColE1-like mobility proteins, no transfer of pAT153 has been observed (Table 1). It can also be seen that pACYC184 behaves as a *nic*<sup>-</sup> plasmid. Similarly, ColE1 deletions lacking *HaeII*-C are non-mobilisable because they lack *nic*<sup>15</sup>. The increased gene-dosage effects resulting from the increased copy number of the ColE1 deletion derivatives have also been detected by the increased antibiotic resistance conferred on plasmid-containing cells and by the increased frequency of spontaneous colicin production by cells containing colicin-producing copy mutants (A.J.T., unpublished).

**Table 1** Plasmid content of cells containing Cm<sup>r</sup> ColE1 deletion mutants

| ColE1 derivative | Size (kilobases) | ColE1 plasmid content (as % of chromosomal DNA) when: |              |                |                 |                 |                 |                 |                 |
|------------------|------------------|-------------------------------------------------------|--------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  |                  | No other plasmid present                              | ColK present | pSC101 present | pDS4101 present | pDS4155 present | pDS4151 present | pDS4152 present | pDS4156 present |
| ColE1            | 6.3              | 3.8 (0.92)                                            | —            | —              | —               | —               | —               | —               | —               |
| pAT201           | 7.8              | 2.8 (0.44)                                            | —            | —              | —               | —               | —               | —               | —               |
| pAT202           | 5.4              | 13.9 (2.1)                                            | 2.1 (0.31)   | —              | —               | —               | —               | —               | —               |
| pAT203           | 7.8              | 4.5 (0.96)                                            | —            | —              | —               | —               | —               | —               | —               |
| pAT204           | 6.9              | 16.5 (1.2)                                            | 2.9 (0.53)   | 13.0 (0.98)    | 0.7 (0.10)      | 12.0 (1.4)      | 2.5 (0.38)      | 20.9 (3.1)      | 0.8 (0.20)      |
| pAT205           | 6.2              | 17.7 (2.7)                                            | 1.4 (0.60)   | —              | 1.4 (0.53)      | 13.7 (0.96)     | 0.8 (0.23)      | 15.0 (1.8)      | 0.5 (0.15)      |
| pAT273           | 3.9              | 20.5 (1.8)                                            | 1.9 (0.32)   | 13.6 (1.6)     | —               | —               | —               | —               | —               |
| pAT277           | 4.3              | 20.6 (2.0)                                            | 1.4 (0.28)   | 19.9 (2.2)     | 0.5 (0.21)      | 12.2 (1.6)      | 2.1 (0.29)      | 18.8 (1.1)      | 0.5 (0.24)      |

Plasmid content values are means of at least three determinations carried out as described in Fig. 2 legend. These values can be easily converted to copy number per genome equivalent ( $3.8 \times 10^3$  kilobase pairs). Values in parentheses are standard deviations. —, Not determined.



**Fig. 2** Copy-number determination. Representative 1% agarose gel electrophoresis of total cell lysates. Stationary phase cells (about one colony equivalent) from either selective agar or a liquid culture were resuspended in 250  $\mu$ l of 40 mM Tris acetate, 20 mM sodium acetate, 1 mM disodium EDTA (pH 8.2) electrophoresis buffer containing 1% SDS, 2% Ficoll and 0.01% bromophenol blue. After heating at 65°C for 30 min and vortex mixing, 50  $\mu$ l each of undiluted and 10-fold diluted sample were applied to alternate gel wells. After running (50 V for 4 h) and staining with ethidium bromide (0.5  $\mu$ g ml<sup>-1</sup>, 60 min), the UV-fluorescence of the gel was photographed using a range of exposures. Microdensitometer tracings of suitable photographic negatives allowed the relative proportions of plasmid and chromosomal DNA to be determined for each lysate. This gel shows the results of copy-number complementation by ColK derivatives. *a*, pDS4156 + pAT205; *b*, pDS4152 + pAT205; *c*, pAT205 alone. The fainter alternate tracks are the respective 10-fold diluted samples. Although most of our copy-number determinations were made on stationary phase cells, the same relative differences between ColE1 and its copy mutants were observed in exponentially growing cells.

## Excision sequences in the mitochondrial genome of yeast

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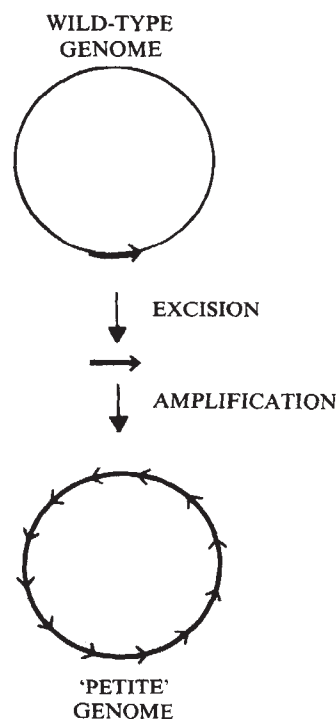
It is well established that spontaneous cytoplasmic 'petite' mutants of *Saccharomyces cerevisiae* have mitochondrial genome units in which an excised segment of the parental wild-type genome has been tandemly amplified (Fig. 1), so that the excised segment becomes the repeat unit of the petite genome; the latter may in turn undergo further deletions leading to secondary petite genomes having shorter repeat units (see ref. 1 for a brief review). Recent investigations<sup>2</sup> on the mitochondrial genomes of several spontaneous petite mutants have shown that frequently the ends of the excised segment correspond to short sequences of the wild-type genome which are extremely rich in GC, the GC clusters; alternatively, they seem to be located in the long AT-rich stretches, the AT spacers, which form at least half of the genome. As sequence repetitions have been demonstrated in both GC clusters and AT spacers<sup>2-5</sup>, it is very likely that excision takes place by a mechanism involving illegitimate site-specific recombination events between homologous sequences, as previously postulated<sup>1</sup>. We show here that the sequences involved in the excision of a particular spontaneous petite genome are direct nucleotide repeats located in the AT spacers.

The presumptive replication repressor described above seems not to be intimately involved in determining plasmid incompatibility<sup>16,17</sup>, the phenomenon that prevents two closely related replicons stably coexisting within a cell. ColE1 and ColK have cross-reacting repressors, but are completely compatible, whereas two ColE1 derivatives lacking *Hae*II-C are not stably co-maintained. Moreover, other ColE1 sequences are involved in determining plasmid copy number. For example, an insertion of some 30 base pairs in part of the ColE1 region essential for autonomous replication results in a copy-number phenotype<sup>4,5</sup>. The RNA transcript of the region seems to be important in specifying copy number<sup>5</sup>. It will be of interest to study the interactions of the two types of copy-number mutant.

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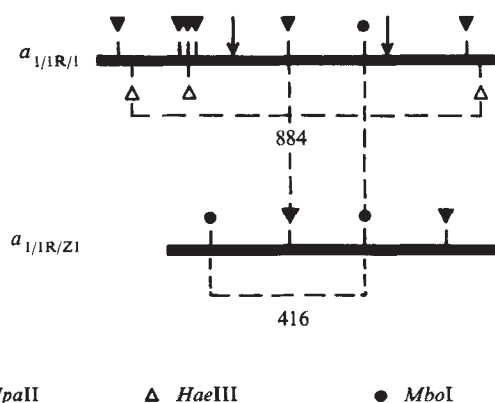
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**Fig. 1** Scheme of the process leading to the formation of spontaneous petite genomes. A segment of a mitochondrial genome unit from wild-type yeast cells is excised and tandemly amplified to yield the mitochondrial genome unit of a petite mutant. The excised segment from the wild-type genome becomes the repeat unit of the petite genome. This may in turn undergo further deletions leading to secondary petite genomes having simpler repeat units.





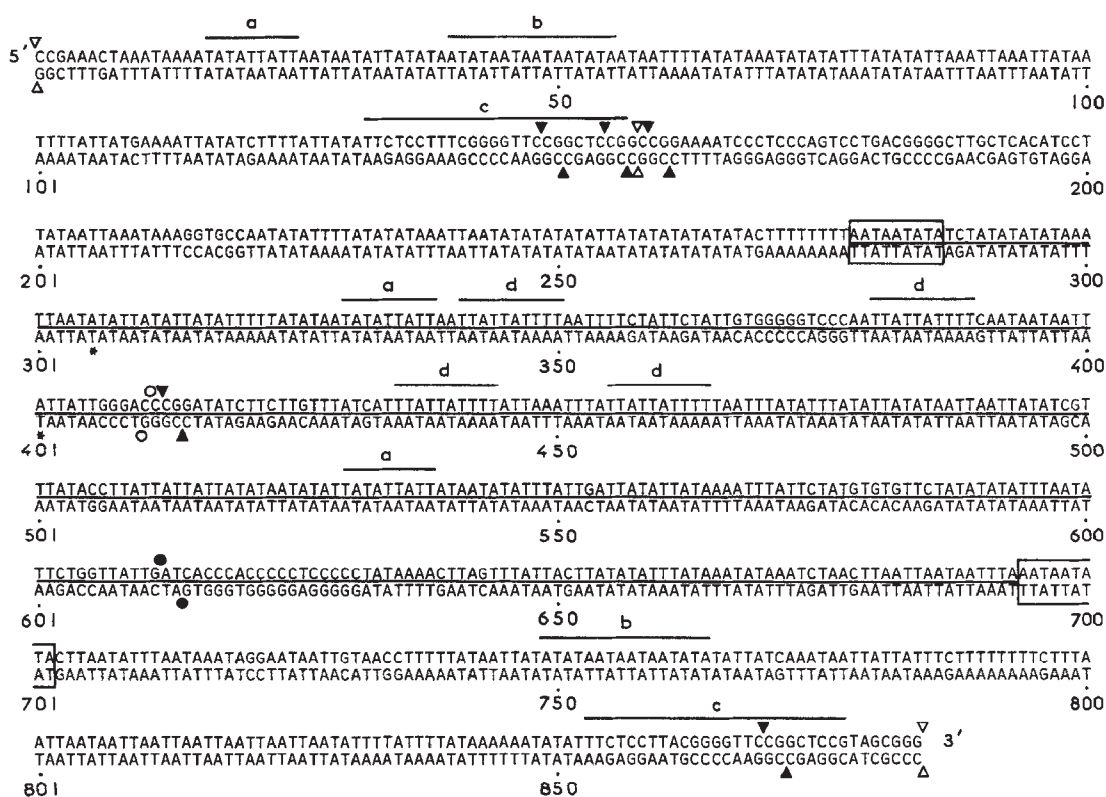
**Fig. 2** Restriction enzyme maps of the repeating units of the mitochondrial genomes of two spontaneous petite mutants. The molecular weights of the repeat units are indicated (horizontal broken lines), along with the positions of *HaeIII* ( $\Delta$ ), *HpaII* ( $\nabla$ ) and *MboI* ( $\bullet$ ) restriction sites. The vertical broken lines indicate corresponding restriction sites in the repeat units of the two genomes. Arrows indicate the approximate positions of excision sites as obtained from the data of Fig. 3.

Our approach was to sequence the repeat unit of the mitochondrial genome of a spontaneous petite mutant,  $a_{1/1R/1}$ , which was supposed to contain the repeat unit and the flanking sequences of another spontaneous petite mutant,  $a_{1/1R/Z1}$ . Both petite genomes had been previously characterised<sup>2</sup>; they originated from the same region of the parental wild-type mitochondrial genome (between map positions 27 and 46, ref. 6) and their restriction maps were known (Fig. 2); the DNA from  $a_{1/1R/Z1}$  hybridised on restriction fragments from wild-type

DNA corresponding to the larger *HaeIII* fragments and to the two largest *HpaII* fragments of  $a_{1/1R/1}$ , and shared a *HpaII*-*MboI* fragment with  $a_{1/1R/1}$ ; finally, the sequence of the repeat unit of  $a_{1/1R/Z1}$  was known<sup>5</sup>.

The nucleotide sequence was determined, using the method of Maxam and Gilbert<sup>7</sup>, on both mitochondrial DNA prepared from  $a_{1/1R/1}$  and its *MboI* repeat unit inserted in the *BamHI* site of pBR322 plasmid and amplified in a *rec<sup>+</sup>* *Escherichia coli* strain (HVC46; C600 *leu<sup>-</sup>*, *thi<sup>-</sup>*, *lac<sup>-</sup>*, *tonA*, *supE44*, *hsdR*, *hsdM*). The same sequence was found in both cases (Fig. 3). The repeat unit of  $a_{1/1R/1}$  is 884 nucleotides long and contains, between positions 278 and 693, a 416-nucleotide segment which is identical to the sequence of the repeat unit of  $a_{1/1R/Z1}$  (ref. 5), except for two base pair changes, which are indicated by an asterisk in Fig. 3. The most important feature of the sequence of  $a_{1/1R/1}$  is that the initial nonanucleotide of the repeat unit of  $a_{1/1R/Z1}$ , AATAATATA, is repeated just after the end of the repeat unit; if allowance is made for one A·T to T·A change, these direct repeats are, in fact, 13 nucleotides long. This situation is reminiscent of those found on both sides of insertion sequence IS1 and of transposon Tn9 in *E. coli*, where two direct repeats of 9 base pairs were found<sup>8,9</sup>. It provides evidence consistent with the excision model proposed previously, and raises the interesting possibility that segments like  $a_{1/1R/Z1}$  can be transposed onto other mitochondrial genome units and contribute to the changes in length observed in genome units belonging to different strains<sup>10</sup>. Incidentally, the data do not allow one to decide whether the repeat unit of  $a_{1/1R/Z1}$  was excised from the parental wild-type genome or from  $a_{1/1R/1}$ , although the latter explanation seems the more likely. This is not an essential point, however, as it is known that the same excision process is operative in wild-type and petite genomes<sup>2</sup>.

Several other features of the sequence of  $a_{1/1R/1}$  are worth mentioning: (1) two direct AT repeats, 16 nucleotides long, containing the nonanucleotides flanking the  $a_{1/1R/Z1}$  sequence,



**Fig. 3** Nucleotide sequence of the repeat unit of the mitochondrial genome of spontaneous petite mutant  $a_{1/1R/1}$ . Cutting sites of *HaeIII* ( $\Delta$ ), *HpaII* ( $\nabla$ ), *MboI* ( $\bullet$ ) and *MboII* ( $\circ$ ) are indicated. The sequence between positions 278 and 693 is identical to that of  $a_{1/1R/Z1}$  (ref. 15) except for asterisked base pairs 306 and 401, which are replaced by G·C and C·G, respectively, in  $a_{1/1R/Z1}$ . Boxed sequences correspond to the nonanucleotides flanking the sequence of  $a_{1/1R/Z1}$ . Lines indicate repeated sequences (see text).

are found at positions 40–55 and 749–764 (these are labelled b in Fig. 3); (2) two GC-rich repeats, 25 nucleotides long with only one A·T to T·A change, are found at positions 132–156 and 853–877 (these are labelled c in Fig. 3); the first one is part of a 65-nucleotide stretch very rich in GC (GC = 65%), which accounts for the higher GC level of  $a_{1/1R/1}$  (GC = 16.2%) compared with  $a_{1/1R/Z1}$  (GC = 14.2%); (3) a 20-nucleotide sequence formed by five repeats of the sequence TTAA is found at positions 809–828; (4) symmetrical sequences are found in the GC cluster at position 150, in agreement with previous predictions<sup>10</sup> and findings<sup>3,4</sup>; (5) other sequence features of  $a_{1/1R/1}$  are common with  $a_{1/1R/Z1}$  and have already been mentioned<sup>5</sup>; the most remarkable one is a 47-nucleotide stretch (369–415) formed by a palindrome 23 nucleotides long (378–400) and a small symmetrical sequence TTATT (402–406), which are flanked by two symmetrical GC clusters (369–377 and 407–415).

The main conclusion of the present work is that crossing-over involving short homologous sequences is indeed the most likely mechanism underlying the excision of spontaneous petite genomes. Sequences used in the excision of other spontaneous petite genomes are being investigated in our laboratory. Some of the genomes under consideration are also encompassed by  $a_{1/1R/1}$ , and it will be interesting to see which other sequences of  $a_{1/1R/1}$  are involved in excision. We expect to find among them some or the other repeats of  $a_{1/1R/1}$ .

Another point of interest is that the presence of direct repeats in its sequence has not caused any instability of the  $a_{1/1R/1}$  repeat unit as propagated in a *rec<sup>+</sup>* *E. coli*; it should be stressed, however, that monomers of the repeat unit were cloned and that oligomers were not tested.

Finally, an inspection of the sequence of  $a_{1/1R/1}$  fails to reveal the presence of any gene, a conclusion confirmed by the present knowledge of codons used for the synthesis of mitochondrially encoded proteins<sup>4,11</sup> and of tRNA gene sequences<sup>12</sup>. The only function left in this petite genome (as well as in  $a_{1/1R/Z1}$ ) is replication. As the excision process leading to the formation of spontaneous petite genomes is extremely conservative as far as the excised sequence is concerned<sup>2</sup> (in contrast to that induced by ethidium, where sequence rearrangements are frequent<sup>13</sup>), each repeat unit of  $a_{1/1R/1}$  should contain a site used for the initiation of DNA replication. A good candidate is the 80-nucleotide sequence centred around position 412. This sequence, (which contains the remarkable stretch, 369–415, mentioned above), is present not only in the two overlapping genomes  $a_{1/1R/1}$  and  $a_{1/1R/Z1}$ , but also in another one<sup>14</sup>, b, whose 875-nucleotide repeat unit is derived<sup>2</sup> from an entirely different region of the parental wild-type genome (map positions 64–76, ref. 6). If a site for the initiation of DNA replication is present in each repeat unit of these petite genomes, a high replication rate may be expected. This indeed seems to be the case, as the three petites under consideration are 'supersuppressive', namely petites whose mitochondrial genomes compete out those of wild-type cells in crosses and become the only ones transmitted to the progeny<sup>14</sup>.

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## Are snRNPs involved in splicing?

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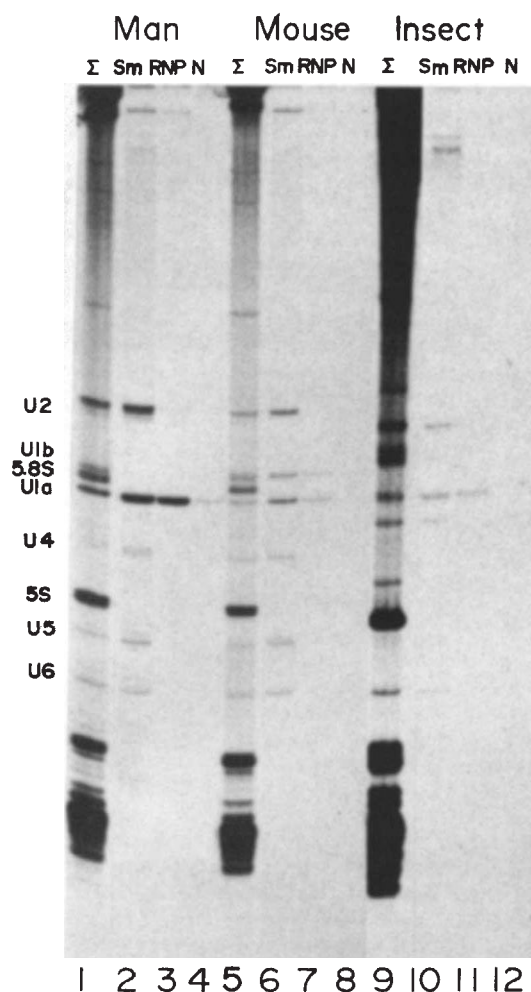
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Discrete, stable small RNA molecules are found in the nuclei of cells<sup>1</sup> from a wide variety of eukaryotic organisms<sup>2</sup>. Many of these small nuclear RNA (snRNA) species, which range in size from about 90 to 220 nucleotides, have been well-characterised biochemically<sup>3–6</sup>, and some sequenced<sup>7,8</sup>. However, their function has remained obscure. The most abundant snRNA species exist as a closely related set of RNA–protein complexes called small nuclear ribonucleoproteins (snRNPs)<sup>9</sup>. snRNPs are the antigens recognised by antibodies from some patients with lupus erythematosus (LE), an autoimmune rheumatic disease<sup>10,11</sup>. Anti-RNP antibodies from lupus sera selectively precipitate snRNP species containing U1<sup>7</sup> and U1b RNAs from mouse Ehrlich ascites cell nuclei, whereas anti-Sm antibodies bind these snRNPs and four others containing U2 (ref. 8), U4, U5 and U6 (ref. 9) RNAs. Both antibody systems precipitate the same seven prominent nuclear proteins (molecular weight 12,000–32,000). All molecules of the snRNAs U1, U2, U4, U5 and U6 appear to exist in the form of antigenic snRNPs<sup>9</sup>. The particles sediment at about 10S and each probably contains a single snRNA molecule. Indirect immunofluorescence studies (refs 12, 13, and unpublished observations) using anti-RNP and anti-Sm sera confirm the nuclear (but non-nucleolar) location of the antigenic snRNPs. Here we present several lines of evidence that suggest a direct involvement of snRNPs in the splicing of hnRNA. Most intriguing is the observation that the nucleotide sequence at the 5' end of U1 RNA exhibits extensive complementarity to those across splice junctions in hnRNA molecules.

If snRNPs participate in RNA processing, they might be expected to be strictly conserved across those higher eukaryotic species possessing interchangeable transcription and mRNA processing systems (refs 14–17 and P. Chambon, personal communication). Figure 1 shows that the antigenic snRNPs are indeed highly conserved from man to insects. The anti-RNP lanes reveal that mouse snRNPs containing the closely related U1a and U1b RNAs<sup>9</sup> are replaced by a single U1-containing snRNP in HeLa and fall armyworm cells. Fingerprints (not shown) indicate that the sequence of HeLa U1 RNA is identical to that of mouse U1a<sup>9</sup> and to that of rat U1a<sup>7</sup>; insect U1 RNA has a quite different fingerprint, although it is comparable in size to the mammalian U1. Likewise, when anti-Sm antibody is used, four additional HeLa or insect cell snRNPs that contain RNAs very close in mobility to mouse U2, U4, U5 and U6 are precipitated; again, fingerprints of the HeLa U2, U4, U5 and U6 RNAs are identical to those of mouse (not shown). Also, human antibodies precipitate snRNPs from the nuclei of frog, chicken and sea urchin cells, indicating high conservation of both RNA and protein components of snRNPs in these species as well. No cross-reacting material is detected in tobacco cell, yeast, *Dictyostelium discoideum* or *Escherichia coli* extracts.

A second prediction concerning snRNP involvement in RNA biogenesis is that snRNAs should be present in highest abundance in metabolically active cell types. Figure 2 compares nuclear extracts from liver and from red blood cells (RBC) of chickens. The sucrose gradient profiles (Fig. 2a) showing extracts from equal numbers of the two types of nuclei reveal fewer than one-tenth as many 30S hnRNP particles (the highly conserved ribonucleoprotein complexes that bind hnRNA in the nuclei of higher eukaryotes<sup>18–20</sup>) and much less RNA sedimenting in the 10S region in the case of the RBC. Also, gel analysis of





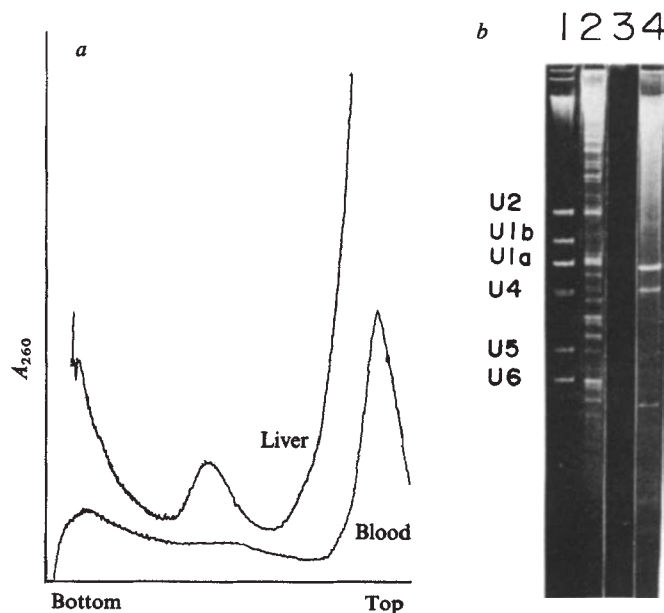
**Fig. 1** Comparison of snRNPs from different species. Small RNAs from cell sonicates or immune precipitates from HeLa (lanes 1–4), Ehrlich ascites (lanes 5–8) and fall armyworm (lanes 9–12) cells were prepared as previously described<sup>9</sup> using Pansorbin to obtain the antigen-antibody complexes. The gel contained 10% polyacrylamide, 7 M urea, 1 mM EDTA, 50 mM Tris-borate pH 8.3 and was 400 × 200 × 0.5 mm. Lanes 1, 5 and 9 show small RNAs in whole cell extracts; 2, 6 and 10 show typical anti-Sm patterns; while 3, 7 and 11 show typical anti-RNP patterns. Lanes 4, 8 and 12 are controls using normal serum.

nuclear RNAs reveals snRNAs in both cases, but about 25-fold fewer in liver than in RBC (Fig. 2b, compare lane 2 with lanes 3 and 4). Treatment of the chicken with phenylhydrazine, which raised the level of erythroblasts to about 25% of the total RBC, altered the snRNA gel pattern (not shown) to that characteristic of liver cells (Fig. 2b, lane 2). Thus, liver cells, which actively synthesise mRNA, have both higher amounts and a slightly different population of snRNAs than the cryptic nuclei of biosynthetically inactive erythrocytes. Were the result otherwise, it would constitute evidence against snRNP participation in RNA processing.

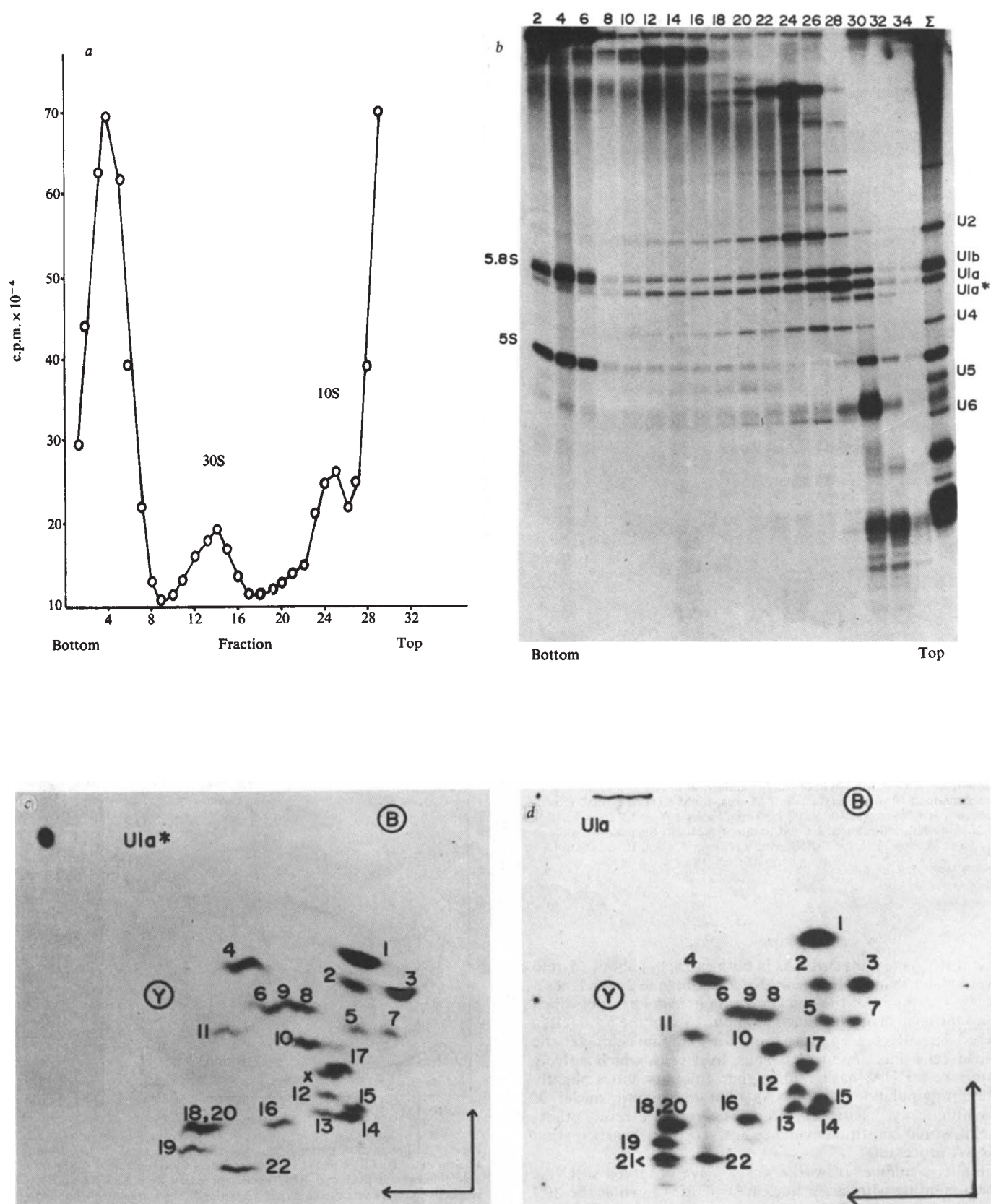
Finally, a number of workers<sup>4,21–24</sup> have observed snRNAs co-sedimenting with larger nuclear structures, such as the 30S particles that bind hnRNA. We have argued<sup>9</sup> that the snRNAs U1, U2, U4, U5 and U6 are not structural components of 30S hnRNP. Yet, as shown in Fig. 3a, b, on sucrose gradient fractionation of a nuclear extract prepared from Ehrlich ascites cells, antigenic snRNPs containing these snRNA species do sediment in the 30S region of the gradient. An RNA-labelled U1a\*, however, appears only in the 10S region where the free snRNP particles sediment. Fingerprint analysis (Fig. 3c) reveals that

U1a\* is identical to U1a (Fig. 3d) except that it lacks the 5' trimethylated cap moiety plus six additional nucleotides from the 5' end of the U1a sequence. Further, U1a\* is not present in fresh extracts and the RNA-protein complex containing U1a\* is fully antibody precipitable, strongly suggesting that U1a\* is an *in vitro* degradation product of U1a. Thus, the absence of a short region at its 5' terminus seems to prevent the U1a-containing snRNP from co-sedimenting with larger structures containing hnRNA.

It is interesting that several of the residues missing in U1a\* fall within a region of U1a which is extensively complementary to sequences across splice junctions in hnRNAs (Fig. 4). DNA sequences corresponding to the ends of 43 introns were analysed (Table 1). Redundant sequences (those which are identical and occur in the same position on homologous RNAs) were discarded, leaving 26 unique sequences at the 5' ends and 31 unique sequences at the 3' ends of introns. The two consensus splice junction sequences are presented in Fig. 4a; in each position, the frequency of occurrence of the most common base is indicated by underlining as described in the legend. Potential base-pairing interactions between these regions and the nucleotide sequence at the 5' end of U1a<sup>7</sup> or U1b RNA<sup>9</sup> are also shown. Note that the complementarity of U1 to the consensus sequence at the 5' end of introns involves eight contiguous residues, plus an adjacent nucleotide (three bases before the splice point) which is generally either C or A. (It seems reasonable to consider that the  $\hat{c}$  position is also complementary since A·G base pairs are observed where the two strands of an RNA helix diverge in yeast tRNA<sup>Phe</sup> (ref. 25).) Of the 26 5' junction sequences, all but one has the dinucleotide GU immediately adjacent to the splice point and at least five (usually six) bases of the consensus sequence. Similarly, at the 3' end of introns, all but one of the 31 unique sequences has the dinucleotide AG, and all



**Fig. 2** Sedimentation patterns and gel analysis of small RNAs from nuclear extracts of liver and red blood cells of an adult chicken. *a*, Comparable numbers of liver and RBC nuclei (prepared as in refs 18 and 9, respectively) were extracted with pH 8.5 buffer<sup>18</sup>. The extracts were applied to 15–30% sucrose gradients and centrifuged for 17 h at 4 °C and 23,000 r.p.m. in an SW 41 rotor. SDS polyacrylamide gel electrophoresis<sup>9</sup> confirmed that the 30S peak contains primarily the prominent proteins of the 30S hnRNP<sup>18–20</sup>. *b*, RNAs obtained by phenol extraction were analysed by electrophoresis as in Fig. 1. Lane 1, marker Ehrlich ascites snRNAs selected by immunoprecipitation with anti-Sm antibody. Lane 2, total RNA from an extract of chicken liver nuclei. Lane 3, total RNA from an extract of an equivalent number of chicken RBC nuclei. Lane 4, total RNA from an extract of chicken RBC nuclei; the amount of extract loaded represents a 25-fold increase in number of nuclei over that shown in lanes 2 and 3.



**Fig. 3** Location and identification of snRNAs after sucrose gradient fractionation of a nuclear extract. *a*, Nuclei were prepared from  $^{32}\text{P}$ -labelled Erlich ascites cells, extracted in high pH buffer<sup>9</sup> and the extract applied to a sucrose gradient as in Fig. 2*a*, *b*. The lanes in the gel (run as in Fig. 1) correspond directly to the numbered fractions in the gradient. Direct phenol extraction and prior immunoprecipitation with anti-Sm serum revealed the same distribution of snRNAs. Some contaminating ribosomes appear near the bottom of the gradient, but SDS polyacrylamide gel analysis reveals that the 30,000–40,000 MW 'core proteins'<sup>18–20</sup> of the hnRNP predominate in the 30S region. Indicated RNA species were identified by fingerprint analysis. *c*, T1 RNase fingerprint of U1a\*. The numbering system corresponds to ref. 7. (Y) and (B) indicate the positions of the blue and yellow dyes in first (horizontal) and second (vertical) dimension. Spot 21 is the 5' end oligonucleotide. *d*, T1 RNase fingerprint of U1a. Spot X is the remnant of the 5' oligonucleotide.



Table 1 Intron-exon boundary sequences

| Gene                     | 5'Exon                                    | Intron                                     | Exon 3' |
|--------------------------|-------------------------------------------|--------------------------------------------|---------|
| Rat insulin              | CAGGUAUGU                                 | ... CUAUCUCCAGG                            | 36 +    |
| Rat insulin              | AAGGUAAGC                                 | ... CUCCUGGCAGU                            | 36      |
| Rat insulin              | CAGGUAUGU                                 | ... CUAUCUCCAGG                            | 36      |
| γ1 chain (newborn mouse) | CAGGUAUGU                                 | ... UUUUCUUGUAGC                           | 37      |
| γ1 chain (newborn mouse) | UUGGUGAGA                                 | ... UCUCUCCACAGU                           | 37      |
| γ1 chain (newborn mouse) | CAGGUAAGU                                 | ... UUCAUCCUAGU                            | 37      |
| γ1 chain (newborn mouse) | AAGGUGAGA                                 | ... CCCACCCACAGG                           | 37      |
| γ1 chain (mouse myeloma) | UUGAGAGGA                                 | ... UCUCUCCACAGU                           | 38      |
| γ2 chain (mouse myeloma) | CAGGUAAGU                                 | ... UUCAUCCUAGU                            | 38      |
| γ1 chain (mouse myeloma) | AAGGUGAGA                                 | ... CUCACUCACAGG                           | 38      |
| γ1 chain (mouse myeloma) | CAGGUCAGC                                 | ... CCUGUUUGCAGG                           | 38      |
| γ1 chain (mouse myeloma) | CAGGUCAGC                                 | ... UCUGUUUGCAGG                           | 38      |
| γ1 chain (mouse myeloma) | UAGGUGAGU                                 | ... UCAUCCUGCGGC                           | 38      |
| γ2 Chain                 | AACGUAAGU                                 | ... UCCUCCUCACAGG                          | 39      |
| λ1 Chain                 | AACGUAAGU                                 | ... UCCUCCUCACAGG                          | 40      |
| λ1 Chain                 | AACGUAAGU                                 | ... UCCUCCUCACAGG                          | 40      |
| κ Chain                  | AACGUAAGU                                 | ... UCCUCCUCACAGG                          | 41      |
| κ Chain                  | AACGUAAGU                                 | ... UCCUCCUCACAGG                          | 41      |
| κ Chain                  | AAGGUUAAA                                 | ... UCCACUCCUAGG                           | 41      |
| κ Chain                  | CAGGUUGGU                                 | ... UCCUCCUCACAGG                          | 41      |
| κ Chain                  | AGGGUGAGU                                 | ... UAUUCCACAGC                            | 41      |
| κ Chain                  | CAGGUUGGU                                 | ... CAUUUUCACAGG                           | 42      |
| Mouse β-globin           | AGGGUGAGU                                 | ... UUUUCCUACAGC                           | 43      |
| Mouse β-globin           |                                           | ... UUUUCCUACAGC                           | 43      |
| Rabbit β-globin          |                                           | ... UCCUCCACAGC                            | 43      |
| Rabbit β-globin          |                                           | ... CUUCUCCGACG                            | 43      |
| Human β-globin           | AAGGUAGGC                                 | ... GUUUGCUCUAGA                           | 43      |
| Human δ-globin           | AAGGUGAGC                                 | ... UUCAUUACAGG                            | 44      |
| Chicken ovalbumin        | CAGGUACAG                                 | ... UUCUUAUUCAGU                           | 45      |
| Chicken ovalbumin        | CCAGUAAGU                                 | ... UUGCUUUACAGG                           | 46      |
| Chicken ovalbumin        | AUGGUAAGG                                 | ... CAUUCUUAAAGG                           | 46      |
| Chicken ovalbumin        | GAGGUAUUAU                                | ... UGUUCCUCCAGC                           | 46      |
| Chicken ovalbumin        | CAGGUAUGG                                 | ... UUUCCUUGCAGC                           | 46      |
| Chicken ovalbumin        | AAGGUACCU                                 | ... UUUUAUUUCAGG                           | 47      |
| SV40 late mRNAs          | AAGGUUCGU                                 | ... UUUUAUUUCAGG                           | 48, 49  |
| SV40 late mRNAs          | CUGGUAAGU                                 | ... UUUUAUUUCAGG                           | 48, 49  |
| SV40 late mRNAs          | CUGGUAAGU                                 | ... UUUUAUUUCAGG                           | 48, 49  |
| SV40 late mRNAs          | CUGGUAAGU                                 | ... UUUUAUUUCAGG                           | 48, 49  |
| SV40 early mRNAs         | AAGGUAUUU                                 | ... GUGUAUUUUAGA                           | 48, 49  |
| SV40 early mRNAs         | GAGGUAUUU                                 | ... GUGUAUUUUAGA                           | 48, 49  |
| Polyoma late mRNAs       | CAAGUAAGU                                 | ... UAUUCCUCCUAGG                          | *       |
| Polyoma late mRNAs       | CAAGUAAGU                                 | ... UUUAAUUCUAGG                           | *       |
| Polyoma late mRNAs       | CAAGUAAGU                                 | ... UCUAUUUUAAGA                           | *       |
| Silk fibroin             | CAGGUGAGU                                 | ... UUUUGUUUCAGU                           | 50      |
| Consensus                | 36 sequences,<br>26 unique<br>A CAGGUAAGU | 37 sequences,<br>31 unique<br>UYUYUYU CAGG |         |

Sequences at splice junctions. The 36 'donor' (5' end) sequences and 37 'acceptor' (3' end) sequences represent 43 possible splicing events (each event is depicted on a separate line). Underlined sequences are redundant (either because they are identical to a homologous region or because they represent an alternative splice involving the same region) and were not included in our tabulation. Also not included are sequences at late adenovirus mRNA splice junctions as these are proposed to be recognised by VA RNA<sup>26</sup>.

\* R. Kamen and B. Griffin, personal communication.

† Refs.

a

hnRNA consensus sequences:

5' ... AG | GUAAGU ... UYUYUY X CAG | G ... 3'  
exon | intron intron | exon

U1 RNA:

complementary to 3' end junctions  
m<sub>3</sub><sup>2,2,7</sup>GpppA<sub>m</sub>ACUUA<sup>†</sup>CCUGGCAGGAGAU...3'  
complementary to 5' end junctions

b

5' end of hnRNA ——— AGG ——— 3' end of hnRNA  
UYUYUYU X CAGGUAAGU  
3' end of U1 ... AGAGGGACGGUCCAUA<sub>m</sub>U<sub>m</sub>App<sub>m</sub><sup>2,2,7</sup>G  
intron

**Fig. 4** Possible base-pairing interactions between U1 and splice junction consensus sequences. The U1 sequence is from ref. 7. Sequences used to generate the consensus sequences are presented in Table 1. *a*, To appear in the 5' or 3' consensus splice junction sequence, a base must be the most common in that position and occur with a frequency of at least 45%; bases occurring in 75% of the sequences are underlined; those present with 95% or greater frequency are underlined twice. In the position marked †, A occurs 11 times and C 10 times in the 26 unique sequences examined. Y indicates pyrimidines. X marks the location of a single nonconserved base in the 3' consensus sequence. Vertical lines show the most likely location of the splice. The arrow locates the 5' end of U1\*. All bases occurring with a frequency of 45% or greater are shown. *b*, A possible model for alignment of intron-exon boundaries by base pairing between U1 RNA and sequences at both ends of an intron.

snRNP could interact with both junctions simultaneously (Fig. 4*b*). A similar model has been independently proposed suggesting the involvement of an adenovirus-encoded small RNA known as VA in the processing of late adenovirus messages<sup>26</sup>. Although the complementarity between U1 RNA and splice junction sequences is quite striking, the stability of the predicted helices may be insufficient to achieve the fidelity of splicing observed *in vivo*. Thus, it is attractive to invoke RNA-RNA interactions as only one component of the recognition of intron-exon junctions by a particle containing both U1 RNA and proteins.

In summary, the abundance of U1 RNA in active cells (about 10<sup>6</sup> per nucleus), the high conservation of both its sequence and the proteins with which it associates, and its considerable complementarity to splice junctions are all consistent with the idea that U1-containing snRNPs have a key role in eukaryotic mRNA processing. They could either be the splicing enzyme itself (note the example of *E. coli* RNase P, which contains a small RNA complexed with several small proteins<sup>27</sup>) or an auxiliary factor required to align splice junctions. One specific prediction of our hypothesis is that avian and insect U1 RNAs should possess 5' end sequences similar to mammalian U1 as the splice junction sequences determined for the chicken ovalbumin and silkworm fibroin genes show the same degree of complementarity to mammalian U1 as do mammalian splice junction sequences (see Table 1). Likewise, it should be possible to demonstrate direct interaction between a U1-containing snRNP and splice junction sequences in a particular hnRNA. Finally, the introduction of antibodies into living cells or *in vitro* splicing systems<sup>28,29</sup> may result in the inhibition of one or more specific steps in the RNA processing pathway. Experiments relating to this are now underway.

can make at least six (usually eight or nine) of the 11 possible base pairs with U1 RNA.

The complementarity of the U1 sequence to splice junctions in mRNA precursors suggests how hnRNA exons separated by an intervening sequence could be brought into proper orientation for splicing. Because U1 RNA is capable of forming Watson-Crick base pairs with a significant number of residues that lie within the two ends of an intron, a single U1-containing

The fact that the other closely related snRNPs (containing U2, U4, U5, or U6 snRNAs) possess common antigenic determinants suggests that they may function comparably to the U1-containing snRNP in RNA processing; different RNA sequences at their 'active sites' could facilitate more precise recognition of variant splice junction sequences in hnRNA or in other RNA molecules. Alternatively, the additional related snRNPs could participate in the transport of RNA molecules from the cell nucleus, as is suggested by the finding that splicing and export seem to be inseparably linked for those hnRNAs that undergo splicing<sup>30,31</sup>. Whatever the outcome, it seems reasonable to investigate further the proposal<sup>26,32-35</sup> that RNA-RNA interactions provide specificity for the precise editing of eukaryotic transcripts; this possibility seems especially attractive when one considers the central role of base pairing interactions in other essential processes (for example, transcription and translation) that developed at an early stage in evolution.

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## Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*

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Plasmids have an important role in the pathogenicity of certain bacterial species, and *Escherichia coli* provides the most complete example of the relationship involved. Enterotoxigenic strains of *E. coli*, in addition to producing heat-stable and/or heat-labile enterotoxins, may also produce a haemolysin and fimbriate cell surface antigens which facilitate the adherence of the bacterial cell to the mucosa of the small bowel. Numerous studies have shown that these properties are plasmid-mediated<sup>1-5</sup> and that the plasmids act in concert to confer on the host bacterium the ability to produce enteric disease in man and in animals. Moreover, studies with invasive strains of *E. coli* have shown that the Col V plasmid, which codes for the synthesis of colicin V, significantly enhances the pathogenicity of its host bacterium<sup>6,7</sup>. Although the relationship between Col V plasmids and virulence is unclear, reports indicate that Col V-containing strains of *E. coli* are better able to survive in the alimentary tract and that colicine V itself inhibits macrophage function<sup>7,8</sup>. It is probable that bacterial virulence is a complex phenomenon involving both chromosomal and plasmid genes. We describe here a virulence plasmid which mediates tissue invasiveness in human pathogenic strains of *Yersinia enterocolitica*.

*Y. enterocolitica* is an ubiquitous organism found primarily in the alimentary tract of warm-blooded animals, including man, and in freshwater lakes and streams<sup>9</sup>. Organisms currently regarded as *Y. enterocolitica* are biochemically and serologically heterogeneous<sup>9,10</sup>. Nevertheless, certain biochemical and serological types of the organism are consistently associated with acute gastroenteritis and mesenteric lymphadenitis in man<sup>9</sup>. *Y. enterocolitica* serotypes 0:3 and 0:9 are the most common cause of human infection in Europe, Japan and Canada, whereas serotype 0:8 strains are responsible for infections in the United States<sup>9</sup>. Most strains of *Y. enterocolitica* associated with human infection produce a heat-stable enterotoxin (ST) and demonstrate tissue invasiveness as determined by penetration of cultured mammalian cells or by invasion of the conjunctival epithelium of guinea pigs (Serény test)<sup>11-14,19,20</sup>.

We examined the role of plasmids in the virulence of a group of serotype 0:8 strains of *Y. enterocolitica*, obtained during an epidemiological investigation of an outbreak of enteric disease involving 218 school children in New York who had consumed contaminated chocolate milk<sup>15</sup>. The group was interesting because it represents epidemiologically related tissue-invasive and non-tissue invasive strains that are otherwise serologically and biochemically identical. We also examined strains isolated from the incriminated chocolate milk, asymptomatic persons, dairy cattle and water collected from a dairy farm. Those from the latter three sources either did not belong to serotype 0:8 and were believed to be unrelated to the disease outbreak: they were included for comparison with the human-pathogenic serotype 0:8 strains. Each strain was examined for heat-labile toxin (LT) and heat-stable toxin (ST) production, tissue invasiveness (TI) and plasmid DNA content.

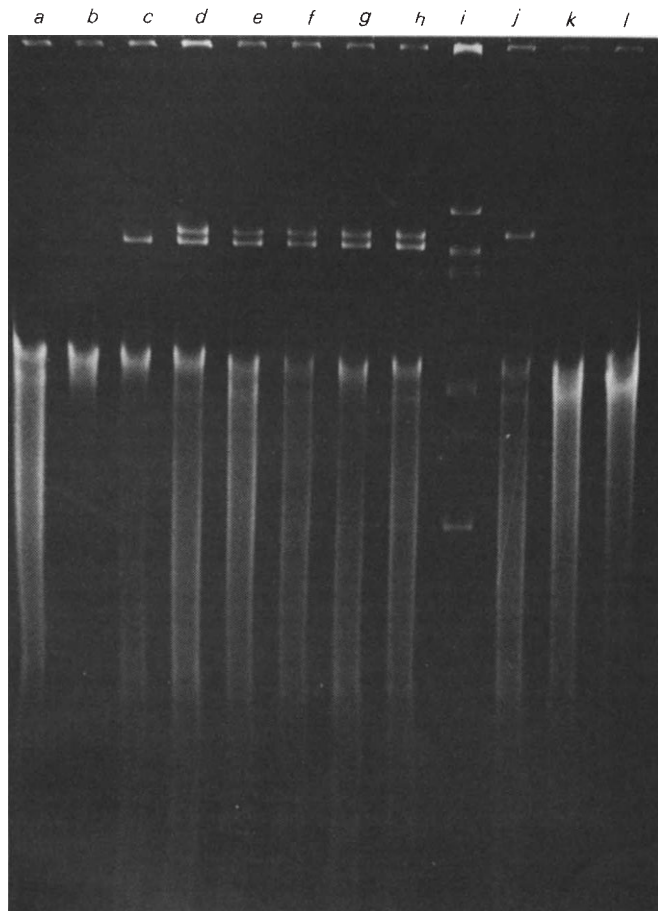
A summary of the results of this examination as well as the source of each strain is given in Table 1. No strains produced LT

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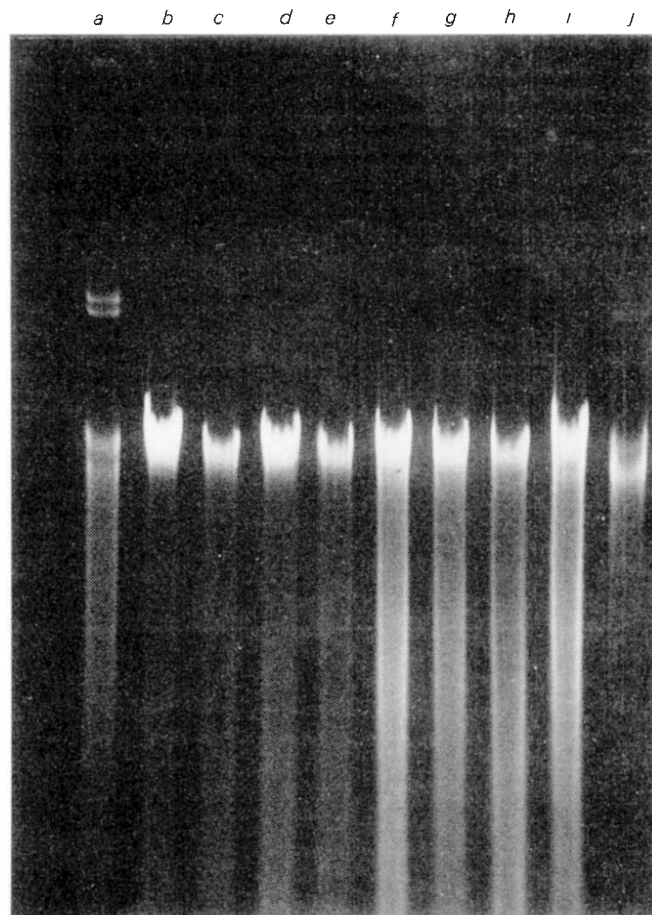


whereas ST production was common. Only serotype 0:8 strains could invade tissue. Serotype 0:8 strains contained either a  $41 \times 10^6$  or both, whereas other strains either did not contain plasmid DNA or they contained plasmids with other molecular weights (Fig. 1). These results suggest that tissue invasiveness is related to the  $41 \times 10^6$ -MW plasmid, because all invasive strains contain the  $41 \times 10^6$ -MW plasmids; in addition, the  $41 \times 10^6$ -MW plasmid is not found in non-invasive strains. There was no apparent relationship between plasmid DNA and ST production. Because most strains of *Y. enterocolitica* produce ST, it is possible that ST production is encoded by chromosomal genes<sup>19,20</sup>.

In an effort to confirm the relationship between tissue invasiveness and the  $41 \times 10^6$ -MW plasmid, we grew strains 54



**Fig. 1** Identification of plasmid DNA in strains of *Y. enterocolitica*. Partially purified plasmid DNA was isolated from strains of *Y. enterocolitica* and subjected to electrophoresis in 0.7% agarose according to the procedure of Meyers *et al.*<sup>24</sup>. Electrophoresis was carried out at 120 V (31 mA) for 3 h through a 15 cm  $\times$  9.6 cm  $\times$  0.6 cm gel. After electrophoresis, the gel was stained for 30 min by immersion in electrophoresis buffer (89 mM Tris base, 2.5 mM Na<sub>2</sub> EDTA, 89 mM boric acid) containing 1  $\mu$ g ethidium bromide per ml. After staining, the gel was rinsed for 1 h under gently running tapwater. The gel was observed and photographed through an orange filter with illumination from a short-wave UV transilluminator. Tracks are as follows: a, strain 62, 18  $\mu$ l partially purified DNA; b, strain 61, 18  $\mu$ l partially purified DNA; c, strain 60, 12  $\mu$ l partially purified DNA; d, strain 59, 12  $\mu$ l partially purified DNA; e, strain 58, 12  $\mu$ l partially purified DNA; f, strain 57, 12  $\mu$ l partially purified DNA; g, strain 56, 12  $\mu$ l partially purified DNA; h, strain 55, 12  $\mu$ l partially purified DNA; i, purified standard plasmid DNA (10  $\mu$ l): upper band, R6-5 (MW  $65 \times 10^6$ ); second band, RP4 (MW  $34 \times 10^6$ ); third band, S-a (MW  $23 \times 10^6$ ); fourth band, pMB9-trimer (MW  $10.5 \times 10^6$ ); fifth band, pSC101 (MW  $5.8 \times 10^6$ ); sixth band, ColEI (MW  $4.2 \times 10^6$ ); j, strain 54, 12  $\mu$ l partially purified DNA; k, strain 53, 18  $\mu$ l partially purified DNA; l, strain 63, 18  $\mu$ l partially purified DNA; m, strain 64, 18  $\mu$ l partially purified DNA.



**Fig. 2** Agarose electrophoresis of DNA from ethidium bromide-cured strains derived from strain 54. Partially purified plasmid DNA was prepared and electrophoresed as described in Fig. 1. Tracks are as follows: a, strain 58, 12  $\mu$ l partially purified DNA; b-i, 25  $\mu$ l partially purified DNA from eight independently isolated strains derived from strain 54 by curing with ethidium bromide at 35  $^{\circ}$ C; j, strain 60, 12  $\mu$ l partially purified DNA.

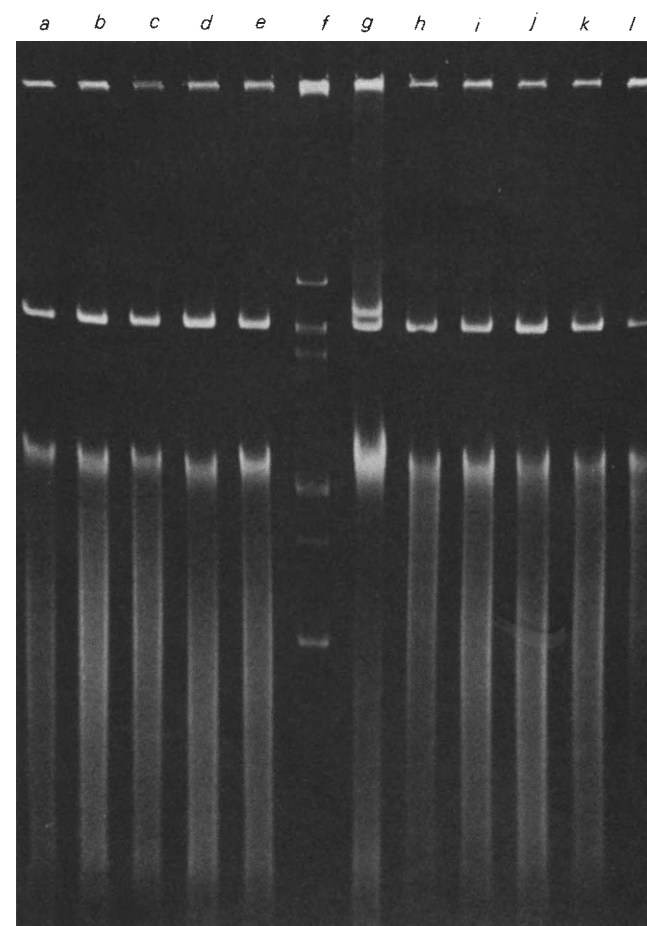
and 58 at 35  $^{\circ}$ C in the presence of the DNA-intercalating dye, ethidium bromide, which is known to inhibit the replication of some plasmids<sup>21</sup>. Single colony isolates, which had arisen from bacteria grown in the presence of ethidium bromide, were tested for ST production and the presence of plasmid DNA. Isolates derived from strain 54 after curing with ethidium bromide no longer contained plasmid DNA (Fig. 2) and were no longer tissue-invasive. Isolates derived from strain 58 after curing with ethidium bromide now contained only the  $35 \times 10^6$ -MW plasmid (Fig. 3) and were no longer tissue-invasive. Even after repeated subculture, the cured strains did not regain the ability to invade tissue. The  $41 \times 10^6$ -MW plasmid could not be detected in the cured strains by either agarose gel electrophoresis or ethidium bromide-caesium chloride buoyant density ultracentrifugation. We found that the  $41 \times 10^6$ -MW plasmid could be cured from strains 54 and 58 simply by growing them at 35  $^{\circ}$ C. Table 2 summarises the results of these curing experiments. From these data, it is clear that the  $41 \times 10^6$ -MW plasmid is efficiently cured simply by growing the host strain at 35  $^{\circ}$ C. These experiments confirm a relationship between tissue invasiveness and the  $41 \times 10^6$ -MW plasmid because loss of the plasmid was always accompanied by loss of tissue invasiveness, regardless of the curing procedure. These results explain earlier observations that some strains of *Y. enterocolitica* lose the ability to invade tissue when grown at 35  $^{\circ}$ C (refs 11, 14).

When  $41 \times 10^6$ -MW plasmid-cured derivatives of strains 54 and 58 were tested for the ability to produce ST, those treated at 35  $^{\circ}$ C with ethidium bromide had become temporarily ST negative, while 35  $^{\circ}$ C alone had not affected ST activity. ST negative strains usually regained ST activity after repeated subculturing.

This further supports the hypothesis that ST production in *Y. enterocolitica* is encoded by chromosomal genes.

One of the serotype 0:8 strains, strain 60, was isolated from a symptomatic child, yet lacked both the plasmid of MW  $41 \times 10^6$  and the ability to invade tissue, possibly because this strain was subcultured at 37 °C before inclusion in our study. The other strains used were not subcultured above 26 °C. Strain 54, a tissue-invasive isolate from the incriminated chocolate milk contains only the plasmid of MW  $41 \times 10^6$ . Presumably, the contaminated chocolate milk could have contained as many as four different types of serotype 0:8 *Y. enterocolitica*: those containing no plasmid DNA, those containing both the  $35 \times 10^6$ -MW and  $41 \times 10^6$ -MW plasmids, and those containing either of the two plasmids. We believe that a strain containing both plasmids was responsible for the disease outbreak, because such strains were isolated independently from the faeces of several symptomatic individuals. At this time, it is not known if the plasmid of MW  $35 \times 10^6$  has a role in human pathogenicity, however, the plasmid of MW  $41 \times 10^6$  is clearly associated with tissue invasiveness as determined by the Serény test.

We believe this is the first report of a plasmid that mediates tissue invasiveness in a member of the Enterobacteriaceae. These plasmids may also be present in other enteric pathogens, notably *E. coli* because *Y. enterocolitica* and *E. coli* are known to exchange plasmid DNA<sup>22,23</sup>. Furthermore, this report serves to illustrate the value of a molecular and genetic characterisation of plasmid DNA in bacteria isolated during an epidemiological investigation.



**Fig. 3** Agarose electrophoresis of DNA from strains derived from strain 58 by curing with ethidium bromide. Partially purified plasmid DNA was prepared and electrophoresed as described in Fig. 1. Tracks are as follows: a–e and h–l, 25 µl partially purified DNA from 10 independently isolated strains derived from strain 58 by curing with ethidium bromide at 35 °C; f, purified standard plasmid DNA (10 µl) as described in Fig. 1; g, strain 58, 25 µl partially purified DNA.

**Table 1** Characteristics of strains of *Y. enterocolitica*

| Strain no. | Source             | Serotype | LT* | ST† | TI‡ | Plasmid DNA MW ( $\times 10^6$ ) |
|------------|--------------------|----------|-----|-----|-----|----------------------------------|
| 62         | Cow                | 4, 33    | –   | +   | –   | —                                |
| 61         | Asymptomatic human | 5        | –   | +   | –   | 3.1; 47                          |
| 60         | Symptomatic human  | 8        | –   | +   | –   | 35                               |
| 59         | Symptomatic human  | 8        | –   | +   | +   | 35; 41                           |
| 58         | Symptomatic human  | 8        | –   | +   | +   | 35; 41                           |
| 57         | Symptomatic human  | 8        | –   | +   | +   | 35; 41                           |
| 56         | Symptomatic human  | 8        | –   | +   | +   | 35; 41                           |
| 55         | Symptomatic human  | 8        | –   | +   | +   | 35; 41                           |
| 54         | Chocolate milk     | 8        | –   | +   | +   | 41                               |
| 53         | Asymptomatic human | 5        | –   | –   | –   | —                                |
| 63         | Water              | 6, 31    | –   | –   | –   | —                                |

LT, ST and TI were determined on cultures grown at 26 °C for 48 h (broth cultures were in tryptic soy broth (Difco) with 0.6% yeast extract and agar cultures were on tryptic soy agar, (Difco)).

\* Using the Y 1 adrenal cell method of Sack and Sack<sup>16</sup>.

† Using suckling mice as described by Dean *et al.*<sup>17</sup>.

‡ As determined by the Serény test<sup>18</sup> using a saline suspension ( $10^{10}$ – $10^{11}$  cells per ml) of a 48-h agar culture grown at 26 °C. Approximately 20 µl of this suspension was inoculated into the conjunctival sac.

**Table 2** Curing the  $41 \times 10^6$ -MW plasmid from strains of *Y. enterocolitica*

|                       | TAMU 54 |       |          | TAMU 58 |       |          |
|-----------------------|---------|-------|----------|---------|-------|----------|
|                       | 26 °C   | 35 °C | 35 °C+EB | 26 °C   | 35 °C | 35 °C+EB |
| No. cured†/no. tested | 0/16    | 20/20 | 22/22    | 0/18    | 10/10 | 8/8      |
| No. TI‡/no. tested    | 16/16   | 0/20  | 0/22     | 18/18   | 0/10  | 0/8      |

\* Ethidium bromide (EB), 15 µg ml<sup>-1</sup> in Penassay broth, pH 7.6, 24 h.

† Strains were examined for loss of the  $41 \times 10^6$ -MW plasmid by agarose gel electrophoresis.

‡ Tissue invasiveness (TI) as determined by the Serény test<sup>18</sup>.

We have designated the temperature-sensitive  $41 \times 10^6$ -MW plasmid of strain 54, pDZ19, and the  $35 \times 10^6$ -MW plasmid of strain 60, pDZ20. A physical and genetic characterisation of these plasmids will be published elsewhere.

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## Functional and molecular organisation of an antigen-specific suppressor factor from a T-cell hybridoma

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Thymus-dependent (T) lymphocytes have been shown to have antigen specificity. The antigen receptor on T lymphocytes, in contrast to that on B lymphocytes, does not appear to be of the conventional immunoglobulin (Ig) type. Studies on the antigen-specific factors derived from helper and suppressor T cells (Ts) demonstrated that they possess determinants with antigen binding affinity and products of genes in the H-2 complex (MHC)<sup>1-6</sup>. Furthermore, antibodies against the variable region of Ig heavy chains or idiotypes have been shown to react with T-cell antigen receptors as well as antigen-specific helper and suppressor T-cell factors (TsF)<sup>7-15</sup>. It is, therefore, conceivable that at least two gene products are involved in the structural entity of these receptors: one each coded for by genes in either. To establish the molecular nature of the recognition component of T cells we have used homogeneous TsF from a T-cell hybridoma with a specific function. We report here that the antigen binding and I-J coded molecules on TsF are independently synthesised in the cytoplasm, and are secreted as an associated form of the two molecules; this association is required for antigen-specific suppression of antibody response.

The T-cell hybridomas with specific suppressive activity were established 20 months previously by the fusion of BW5147 (H-2<sup>b</sup>) and enriched Ts of C57BL/6(H-2<sup>b</sup>) mice primed with keyhole limpet haemocyanin (KLH). The hybrids continuously secrete specific suppressor molecules with an antigen binding site and products of genes in the I-J subregion of MHC. The properties and functions of the hybridoma-derived TsF have been reported previously<sup>16</sup>. We attempted to separate the active moiety so as to characterise further the structure of the hybridoma-derived TsF. The suppressive extract or ascitic material from the hybridoma (34S-704) was passed through immuno-adsorbent columns of KLH, anti-I-J<sup>b</sup> and anti-mouse Igs under the same conditions as described previously<sup>16</sup>. The absorbed materials were eluted from columns with glycine-HCl buffer, pH 3.2 at 0 °C. Both effluents and eluates were tested for their suppressive activity. As shown in Table 1, the activity of TsF from the hybridoma was absorbed by KLH and anti-I-J<sup>b</sup> columns but not by the anti-IgS. The activity was successfully recovered in the acid eluate from KLH and anti-I-J<sup>b</sup> columns. These results indicate that TsF in the extracted and secreted materials has both antigen-binding sites and I-J determinants, and can be recovered from either the KLH or anti-I-J<sup>b</sup> column. These two determinants are also demonstrated on the surface of semipurified Ts<sup>15,17</sup> and hybridomas<sup>18</sup>. In contrast, recent reports<sup>9,10</sup> showed that isolated T-cell receptor molecules do not contain detectable MHC gene products. It is, therefore, of interest to determine whether or not the I-J determinant is in fact associated with antigen-receptor of Ts, and if such association is indeed required for suppression. If these two distinct determinants are present on a single molecule, the mixture of

**Table 1** Presence of the associated and non-associated forms of antigen-binding and I-J coded molecules in the extract and lack of the non-associated form in ascites

| Materials added to the culture                                       | Anti-DNP IgG PFC per culture |             |
|----------------------------------------------------------------------|------------------------------|-------------|
|                                                                      | Ascites                      | Extract     |
| None                                                                 | 1,774 ± 182                  | 1,211 ± 269 |
| Unabsorbed                                                           | 313 ± 75                     | 146 ± 113   |
| Absorbed with anti-IgS                                               | 462 ± 164                    | 131 ± 193   |
| Eluted from anti-IgS                                                 | 1,802 ± 440                  | 1,547 ± 172 |
| Absorbed with KLH                                                    | 2,236 ± 174                  | 1,255 ± 465 |
| Eluted from KLH                                                      | 668 ± 237                    | 219 ± 170   |
| Absorbed with anti-I-J <sup>b</sup>                                  | 2,043 ± 258                  | 1,342 ± 21  |
| Eluted from anti-I-J <sup>b</sup>                                    | 580 ± 128                    | 131 ± 193   |
| Combination of materials absorbed with KLH and anti-I-J <sup>b</sup> | 2,118 ± 335                  | 100 ± 94    |

The secreted or extracted materials were obtained from suppressor T-cell hybridoma as described previously<sup>16</sup>. Briefly, the hybrids were made by fusion of AKR thymoma cell line BW5147 with the enriched suppressor T cell of C57BL/6 mice specific for KLH. For hybridisation,  $5 \times 10^6$  enriched suppressor T cells of C57BL/6 origin and  $5-50 \times 10^6$  BW5147 cells were washed twice in serum-free Dulbecco's modified Eagle's medium (DMEM) and pelleted together at 400 g. Polyethylene glycol (2 ml; PEG, molecular weight 2,000)-dimethyl sulphoxide (DMSO) solution (1 weight of PEG plus 1 volume of 15% DMSO-DMEM) was added to the cell pellet and mixed gently. A further 2 ml of 50% (W/V) PEG solution without DMSO was poured into the test tube and the mixture was gently stirred with a Pasteur pipette. The cell suspension was then gradually diluted with 16 ml of serum free DMEM and further diluted with 180 ml of DMEM containing 13% fetal calf serum (FCS). It was then incubated at 37 °C for 3-4 h in 5% CO<sub>2</sub>. After incubation cells were washed four times with DMEM and cultured for 1-2 weeks in HAT medium (RPMI 1640 supplemented with 10% FCS and hypoxanthine, aminopterin, thymidine (HAT)) on Falcon plates (Falcon 3008). Cells grown in HAT medium were collected and stained with anti-I-J<sup>b</sup> anti-serum (B10.A(5R) anti-B10.A(3R)) and fluorescein-conjugated rabbit anti-mouse Igs. The stained cells were then sorted by fluorescence-activated cell sorter, FACS II (Becton-Dickinson), and I-J positive cells were cloned in a multiwell microplate (Falcon 3040) by limiting dilution or single cell manipulation. So far we have established several I-J positive suppressor T-cell hybridomas with specific functions, that is, cell lines 34S-704, 34S-18, 34S-11, 1L-1, 8C-23, and 9F-18, each of which has the same characteristics as the cell line 9F181a described previously<sup>16</sup>. The cell-free extract used was obtained by ultracentrifugation of the frozen and thawed materials of the hybrid cells (34S-704) at 20,000g for 1 h, and the secreted materials were obtained in ascites of hybridoma (34S-704)-bearing F<sub>1</sub> mice. 20 µl of ascites or extracted materials from  $3 \times 10^5$  hybridoma cells were passed through the immuno-adsorbents composed of KLH (5 mg per ml of beads), anti-I-J<sup>b</sup> (B10.A(5R) anti-B10.A(3R)), 1 ml γ-globulin fraction of antiserum per ml of beads, rabbit anti-mouse Igs (5 mg purified anti-mouse Ig antibodies per ml of beads). The beads used were Sepharose 4B prepared as described previously<sup>16</sup>. The materials absorbed by columns were eluted with 0.175M glycine-HCl buffer pH 3.2 followed by an instant neutralisation with 1 M sodium bicarbonate, dialysed for 4-6 h with 0.01 M sodium phosphate-buffered saline pH 7.2 and concentrated by negative pressure. The effluents and the eluates from the columns, and the mixture of the effluents from each column were added to the culture of  $4 \times 10^6$  spleen cells of C57BL/6 mice primed with 100 µg DNP-KLH plus  $1 \times 10^9$  pertussis vaccine in the Mishell-Dutton system (Falcon 3008 plate), in 1 ml RPMI 1640 enriched with 10% FCS in the presence of 0.1 µg DNP-KLH and  $2.0 \times 10^{-5}$  M 2-mercaptoethanol. Anti-DNP IgM and IgG PFC were assayed on day 5 of culture by the method of Cunningham and Szenberg as described previously<sup>3</sup>. Results are expressed as mean numbers of anti-DNP IgG PFC of four cultures ± s.d.

the materials after absorption with the KLH or anti-I-J<sup>b</sup> column should not exert any suppressive activity. On the other hand, if they are on separate molecules and combine when they act on target cells, the mixture should reconstitute the suppressor activity. The mixture of the effluents of ascites from the KLH and anti-I-J<sup>b</sup> columns gave no suppressive effect (Table 1). Unlike ascites, the mixture of the effluents of the extracted material definitely produced a strong suppression, despite the fact that either effluent alone had no detectable activity. These

results indicate that the extract, but not ascites, contains two discrete molecules which would combine to reconstitute suppressor activity. Associated forms of suppressor molecule, however, should be present in the extract, since the activity could be eluted from either KLH or anti-I-J<sup>b</sup> column. The secreted material, however, contains only molecules of the associated form.

These experiments do not, however, exclude the possibility that the suppressor activity in the mixture of the two effluents is caused by the additive effect of residual TsF remaining after absorption, even though the effluents alone did not exert detectable suppressor effects. To eliminate this possibility, the extract was passed through the KLH or anti-I-J<sup>b</sup> column (first column), and the effluent from each of the columns was successively applied to both KLH and anti-I-J<sup>b</sup> columns (second column) (Fig. 1). The effluent from either the KLH or anti-I-J<sup>b</sup> column was tested directly for its suppressive activity. In other experiments, the effluent from the KLH column was reabsorbed with another KLH column to ascertain complete absorption. Similar double absorption tests were performed with anti-I-J<sup>b</sup> column. The effluents, each after absorption twice with the KLH or anti-I-J<sup>b</sup> column were admixed and tested for the suppressor activity. As shown in Table 2, the suppressive activity in the extract was abrogated by absorption with the KLH or anti-I-J<sup>b</sup> column (group III or IV). However, the mixture of the KLH → KLH effluent and anti-I-J<sup>b</sup> → anti-I-J<sup>b</sup> effluent (group V) showed strong suppressor activity. Effluent from the KLH double columns should not contain KLH-binding molecules while I-J molecules are still present. Similarly, after absorption twice with the anti-I-J<sup>b</sup> column, I-J molecules are completely removed leaving KLH-binding molecules in the effluent. Hence, the combination of these two effluents resulted in the mixture of the two molecules which reconstituted the suppressive activity. On the contrary, no suppressive activity was recovered when the effluent from the KLH (or anti-I-J<sup>b</sup>) column was successively absorbed with the anti-I-J<sup>b</sup> (or KLH) column (the mixture of KLH → anti-I-J<sup>b</sup> effluent and anti-I-J<sup>b</sup> → KLH effluent; group VI). These results suggest that antigen-binding and I-J coded

**Table 2** Requirement of the co-existence of I-J bearing and antigen-binding molecules for the manifestation of suppressor function

| Experimental group | Materials after absorption with:<br>first column → second column |                         | Anti-DNP IgG PFC per culture |
|--------------------|------------------------------------------------------------------|-------------------------|------------------------------|
| I                  | None                                                             |                         | 2,824 ± 98                   |
| II                 | Unfractionated                                                   |                         | 525 ± 196                    |
| III                | KLH                                                              | None                    | 1,722 ± 224                  |
| IV                 | Anti-I-J <sup>b</sup>                                            | None                    | 2,923 ± 598                  |
| V                  | KLH                                                              | → KLH                   | combined 383 ± 150           |
|                    | Anti-I-J <sup>b</sup>                                            | → Anti-I-J <sup>b</sup> |                              |
| VI                 | KLH                                                              | → Anti-I-J <sup>b</sup> | combined 2,507 ± 287         |
|                    | Anti-I-J <sup>b</sup>                                            | → KLH                   |                              |

The extracted materials (34S-704) unabsorbed (group II), absorbed with the KLH (group III) or anti-I-J<sup>b</sup> (group IV) column, or the mixture of the effluents from the double columns (groups V or VI) as indicated in Fig. 1 were added to the culture of DNP-KLH primed spleen cells from C57BL/6 mice and tested for their suppressive activity. The assay of anti-DNP IgG PFC is the same as in Table 1. Results are expressed as mean numbers of anti-DNP IgG PFC of four cultures ± s.d.

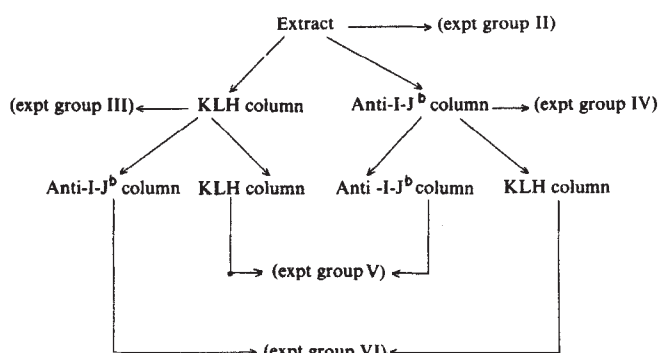
molecules are present independently in the extract, and the association of these two molecules, either of which has no detectable activity by itself, reconstitutes the suppressive activity. Although we have not excluded the possibility that the non-associated form in the extract is due to an imbalance in the synthesis of the two molecules in hybridoma cells, it is concluded that there are at least three molecules in the extracted material: antigen-binding molecule, I-J coded molecule and the associated form of the two molecules. The secreted material consists of the associated form alone. It is, therefore, likely that the two independent molecules in the extract are synthesised in the cytoplasm as a precursor of the suppressor factor which is then secreted after the association.

Our previous studies showed that KLH-TsF specifically suppresses the response of histocompatible spleen cells but not that of allogeneic cells<sup>19</sup>. This genetic restriction is governed by genes in the I-J subregion. The hybridoma-derived TsF also showed rigid genetic restriction<sup>16</sup>. In agreement with this antigen specificity and H-2 restriction of TsF, our present results show firm evidence that the functional structure of TsF is composed of two distinct molecules; one involved in the specific antigen-binding molecule, probably having V<sub>H</sub> structure and the other the I-J coded molecule which may serve as a restricting element in the suppressor cell interaction. A definitive answer must await chemical and physicochemical analysis of the hybridoma-derived suppressor molecule.

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**Fig. 1** Procedures of the absorption of the extract with the KLH and anti-I-J<sup>b</sup> columns. Extract equivalent of  $6 \times 10^5$  hybridoma cells (34S-704) was applied to the KLH or anti-I-J<sup>b</sup> column (first column). The effluent from either the KLH or anti-I-J<sup>b</sup> column was divided into four equal parts, each of which contained the factor equivalent of  $1.5 \times 10^5$  hybridoma cells. Two of these were used as the effluent of group III or group IV as indicated. The other two were subsequently applied to another KLH and anti-I-J<sup>b</sup> column, respectively (second column). The effluent from the KLH → KLH columns and that from the anti-I-J<sup>b</sup> → anti-I-J<sup>b</sup> columns were mixed (group V), as were the effluent from the KLH → anti-I-J<sup>b</sup> columns and that from the anti-I-J<sup>b</sup> → KLH columns (group VI). The unabsorbed (group II), the absorbed (groups III and IV) or the mixture of the effluents absorbed with double columns (groups V and VI) were added to the culture and their suppressive activities were tested in comparison to the response of group I without the factor. The materials of each group should contain the factor equivalent of  $3 \times 10^5$  hybridoma cells.



## REVIEWS

## Dioxin as a health hazard

Alastair Hay

VIETNAM and Seveso are the two principal subjects in this book on dioxin contamination. Vietnam had about 100 kg of 2, 3, 7, 8 — tetrachlorodibenzo-p-dioxin (dioxin) dumped on it in the course of the US military defoliation programme over the country from 1962-1970. Seveso, a small Italian town some fifteen miles north of Milan, was also the hapless victim of dioxin when in July, 1976, a reactor manufacturing trichlorophenol overheated. The reactor contents, including some 1-1.5 kg of dioxin, were vented into the atmosphere and precipitated out over the town and its environs.

The author of this book quite clearly chose Vietnam and Seveso to make the point so aptly summarised in the title. It is Whiteside's thesis that contamination of the environment by dioxin poses a considerable health hazard, and that time to deal with it may be running out.

Much of the material in this book is not new and was first published as two long articles in the *New Yorker*. Whiteside is a staff writer for the magazine and his book is written in the same relaxed style which is a characteristic of this American weekly. The result is that the message is hammered home, all the harder; Whiteside is out to convince readers of the dangers posed by dioxin.

The narrative begins with Vietnam and the military defoliation programme during which over  $11.0 \times 10^6$  gallons of the herbicide Agent Orange (a 1:1 mixture of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid) was sprayed on forests and food crops. This, however, is history. What concerns Whiteside is the fact that 2,4,5-T is still used in the United States. It is 2,4,5-T which contains the dioxin contaminant, carried over from an earlier step in the manufacturing process when trichlorophenol is produced. And dioxin is now known to be a teratogen, mutagen and carcinogen.

Lessons learned in Vietnam about the havoc caused by the herbicides are used by the author to illustrate the risks entailed by the continued use of 2,4,5-T. There are parallels between 2,4,5-T use in Vietnam and in the US, but there is one important difference. The concentration of dioxin

*The Pendulum and the Toxic Cloud: The Course of Dioxin Contamination.* By Thomas Whiteside. Pp. 205. (Yale University Press: New Haven, Connecticut, and London, UK, 1979.) £10.80.

present in 2,4,5-T in the US is probably about 1/100th of that used in Vietnam. Current formulations of 2,4,5-T contain less than 0.1 p.p.m. of dioxin. There are those who argue, however, that even this concentration is high enough to present a health risk. Whiteside takes the same view.

The US Environmental Protection Agency (EPA) is also concerned about 2,4,5-T and in March this year issued a banning order restricting its use. According to the EPA there is a correlation between 2,4,5-T spraying of forests in the state of Oregon and an increase in the rate of spontaneous abortions in the area. The EPA announcement came too late for inclusion in Whiteside's book, nevertheless, the author has captured the changing mood in the US about the wisdom of using 2,4,5-T. In an enlightening anecdote, he reports that "The attitude toward 2,4,5-T has somewhat changed at the Department of Defense it seems. The days are over when during the height of herbicidal operations in Vietnam, an American military spokesman could declare that the relevant herbicidal agents were "not at all harmful to humans or animals" — and illustrate this statement by dabbing on his tongue a bit of liquid from a bottle that sat on his desk".

Seveso is a familiar enough story to readers of *Nature*. When the reactor in the ICMESA chemical plant at Seveso overheated discharging its toxic contents over the surrounding countryside, it caused one of the most serious pollution incidents yet recorded. It also caught the Italian authorities unprepared. Whiteside cites the illogicality of some of the early restrictions on movement of people into and out of the most seriously contaminated zone (Zone A), at Seveso. The authorities, according to Whiteside, "First forbade all entry into it [Zone A] by outsiders and then changed the rules to allow ten persons to enter on weekdays

and twenty on Sundays". "When people in the area heard about these safety measures, some of them just laughed, in spite of the tragedy they were caught up in," one person familiar with the evacuation told me. "They would say to one another, 'so dioxin is twice as dangerous on weekdays as it is on Sundays?'"

Although the Italian authorities perhaps deserved the censure they have received, it should not be forgotten that it was a reactor owned by Givaudan — a Swiss multinational and subsidiary of the giant pharmaceutical company F. Hoffmann La Roche — that caused the accident. Givaudan have always insisted that their reactor design was adequate to meet with foreseeable accidents. Perhaps Seveso was unforeseeable. But it happened, and the reactor design did not meet the situation.

The author does not deal in great detail with the reactor design, preferring instead to concentrate on the consequences of its failure. Whiteside has interviewed many of the scientists and politicians involved with the aftermath of Seveso. Few of them stand out as luminaries. Many of them revealed their party political interest — a loyalty which inhibited the work of the committees concerned with the decontamination and health surveillance programme.

Much of Whiteside's story is concerned with the residents of Seveso. Many lost their livelihood; most received compensation for this loss, but the bitterness still lingers and it comes through in this book.

There are a few points which could give the account a slightly different balance. The author has on several occasions been too selective in his choice of material. For example, there are three studies which now show 2,3,7,8-tetrachlorodibenzo-p-dioxin to be carcinogenic. Two of these agree on the dose level at which dioxin exhibits its carcinogenic effect, the third predicts the chemical to be a carcinogen at dose levels several orders of magnitude lower. Whiteside only publicises the latter. Then there is the case of the link in several Swedish hospitals between hexachlorophene (a bactericide made from trichlorophenol) use and birth

malformations. The author retells this story, but omits to mention that it has not been confirmed, and that the Swedish Health Authority is sceptical about the link.

There is an error in the description of the effect of the 1968 explosion in the trichlorophenol reactor at the UK company, Coalite and Chemical Products Ltd. Whiteside says several workers died after the accident as a result of severe liver damage. This is not the case. Only one worker died, and this was as a direct result of injuries received when the reactor blew up. In Europe, however, workers formerly employed on trichlorophenol

reactors have died, but so far there is no firm evidence to link these deaths with dioxin exposure.

Apart from these criticisms the book makes good reading. It ought to be read by everyone concerned with how relations between the general public and government authorities can go wrong. But the picture is neither as black, nor as clear cut as the author makes out. There are many questions about dioxin still to be answered. □

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## Hippocampal function

T.V.P. Bliss

*The Hippocampus as a Cognitive Map.* By J. O'Keefe and L. Nadel. (Clarendon/Oxford University Press: Oxford, 1978.) £25.

THE hippocampus, a phylogenetically primitive cortical structure long admired by neuroanatomists for the simplicity and regularity of its neural architecture, came to more general notice in the 1950s with the discovery that a bilateral lesion of the hippocampus in man causes severe impairment of memory. This glimpse of the engram aroused great excitement and for the past two decades rats with hippocampal lesions have been among the commoner experimental species in behavioural laboratories around the world. Disappointingly, the benefits of all this effort have not been startlingly apparent. What differences in behaviour can be found between normal and hippocampal rats cannot easily be explained in terms of simple loss of memory. Alternative concepts, such as inhibition and frustration, emerged as candidates for a theory of hippocampal function, but to no very general acclaim. For a time there was a vogue for Keating's hypothesis which allocated to the hippocampus the task of preventing the neocortex from falling into the lateral ventricle and drowning. Meanwhile, and particularly in the past decade, workers from the other end of the neurobiological spectrum — anatomists, physiologists and pharmacologists — have found in the hippocampus a uniquely convenient model for studying synaptic organisation and function in the mammalian cortex, with the result that there is today probably more information, from more sources, about the hippocampus than any other structure in the mammalian brain.

It is an appropriate time therefore for a new look at the problem of hippocampal function. What is needed is a theory which tells us not only what the hippocampus does but also how it does it. This is what O'Keefe and Nadel set out to achieve in their ambitious and stimulating book. According to their theory, the hippocampus is the chartroom of the brain's navigational system, the repository where neural representations of an animal's environment are assembled and stored. The theory derives from the discovery by O'Keefe and Dostrovsky in 1971, that there exist 'place' neurones in the rat's hippocampus which fire only when the animal is at a particular place in the environment; and from a reassessment of the behavioural literature which allows the authors to conclude that it is only in situations where an animal has to identify a place, that hippocampal animals show deficits in behaviour.

The book opens with a discussion of post-Newtonian ideas about space and continues with an illuminating analysis of the ways in which an animal can find its way around space. O'Keefe and Nadel distinguish between two possible navigational strategies (route hypotheses and place hypotheses) based on the two kinds of space which are represented in the brain: relative space, which is centred on the observer, so that spatial relationships between objects in the environment change as the observer moves; and absolute space, which is non-egocentric and items within which are fixed relative to each other. Absolute space can be represented by a map, the neural representation of which, in O'Keefe and Nadel's theory, is located in the hippocampus.

The second section of the book is concerned with the anatomy and physiology of the hippocampus, and opens with a comprehensive account of the afferent and efferent connections of its two principle cell populations, the granule cells of the dentate gyrus and the pyramidal cells of the hippocampus proper. The rather more selective coverage of hippocampal physiology includes a discussion of the

properties of place units and a good account of the mysterious theta waves which can be recorded in the hippocampus of conscious animals during voluntary behaviour such as active exploration. The mechanism responsible for generating theta activity is not well understood, though there is some evidence to support O'Keefe and Nadel's assumption that it plays a role in synchronising the excitability of cell populations in the hippocampus.

The authors then present a model of how the hippocampus might function as a map-generating device. Information about the environment is passed to the granule cells from entorhinal cortex. Each granule cell receives a number of entorhinal fibres, at least two of which, representing some specific combination of sensory stimuli, must be active to fire the cell. The output from each granule cell is fed via synapses *en passage* to a large number of pyramidal cells, whose excitability is modulated by theta waves propagating across the hippocampus as the animal moves through its environment. At a given moment therefore only a proportion of the pyramidal cells receiving impulses from an active granule cell will be at the appropriate phase of theta activity to fire. The set of activated pyramidal cells, each one responding to a particular combination of features in the environment, constitutes the neural representation of place. In order to account for the fact that place units continue to fire if any small subset of the set of stimuli which define the environment is removed, it is assumed that the set of active pyramidal cells become functionally linked together through the strengthening of synaptic connections between them. In the third stage of the model a population of pyramidal cells compares the expected with the perceived environment and fires whenever there is a mismatch, thus providing the trigger for renewed exploration. Although the model makes a valiant attempt to exploit the peculiar characteristics of hippocampal anatomy and physiology, including theta activity and long-term potentiation of synaptic connections, it is nevertheless open to many objections. Much of its anatomical substrate is speculative at best, and in the several hippocampal pathways in which the phenomenon has been demonstrated, long-term synaptic potentiation seems to be a function of activity in afferent fibres alone, rather than of conjoint pre- and post-synaptic activity, as the model requires. A more fundamental objection, and one which the authors don't discuss, is how an animal recognises a familiar place when it is approached from a different direction.

In the third and longest section of the book, O'Keefe and Nadel turn their attention to the behavioural literature, and in particular to the learning ability of rats with hippocampal lesions. Their industry here is overwhelming — there is seemingly nothing which they have not read,



tabulated, critically assessed. The various types of behavioural experiment are carefully explained and for the reader without experience of the psychological literature this is an outstandingly good introduction. The conclusion the authors reach at the end of this exhaustive survey is that, in general, and as the theory would predict, behavioural deficits in hippocampal animals only emerge in tasks which the intact animal solves using place hypotheses; even then the hippocampal animal may be able to compensate by using alternative route-type strategies. This distinction between the use of place hypotheses in normal animals and the compensatory use of route hypotheses in hippocampal animals brings a considerable degree of order to a literature rich in apparently contradictory results. Nevertheless, doubts remain. During the course of their review a barrage of analytical ingenuity is directed against experiments which do not at first sight fit the prediction of the theory. The effect of this is ultimately to raise doubts in the reader's mind about the ability of any experiment, whatever its results, to furnish convincing evidence against the theory.

The final section is taken up with a discussion of the amnesia which results from hippocampal lesions in humans. This is a controversial area with no general agreement about the extent to which amnesia is a failure of recall rather than a failure of storage. O'Keefe and Nadel propose that the human hippocampus is reserved for a context-dependent memory system, having a spatial component equivalent to a cognitive map, which is located in the right hippocampus and a verbal component responsible for memory of connected discourse or narrative, which is represented in the form of a semantic map in the left hippocampus. They conclude that while the use of strategies designed to assist recall can often improve the performance of amnesics in context-free tasks, such as memorising word lists, this is not the case for context-dependent memories: here the loss of memory is total and irrecoverable.

Both authors were at one time students of D.O. Hebb, and their book, though it lacks the stylistic charm of Hebb's monograph *Organization of Behaviour*, follows the same tradition in its attempt to fuse neurobiological and behavioural data into a unified theory of function. The verdict on the theory must, at the moment, be 'unproven'. But whether or not the theory survives, O'Keefe and Nadel have in this book provided a solid basis on which the question of hippocampal function can, at last, be sensibly debated; on that account alone their book is a very considerable achievement. □

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## Nuclear and reactor physics concepts

Malcolm C. Scott

*A Guidebook to Nuclear Reactors.* By A.V. Nero. (University of California Press: Berkeley, Los Angeles and London, 1979.) Hardback £17.50; paperback £5.95.

ALMOST without exception, nuclear power programmes throughout the world have been subjected to critical, and often hostile, scrutiny. There seems to be no single reason for these examinations; certainly, it is not related to the actual safety record of nuclear power reactors, as most re-appraisals predate the accident at the Three Mile Island pressurised water reactor plant at Harrisburg, Pennsylvania. Rather, the opposition to nuclear power has centered on a number of issues. The first of these is the relationship between nuclear power and the spread of nuclear weapons; the second relates to the problem of radioactive waste disposal and the associated question of whether or not we know enough about the effects of radiation; and the third is concerned not with nuclear power *per se*, but with the role of high technology in society.

To an interested but uninformed spectator some of the confrontations which have occurred must have seemed baffling. However, the worst aspect of this is possibly not the fact that 'experts' frequently hold diametrically opposite views but, rather, the fact that, as it is high technology, the whole field of nuclear power and radioactivity is relatively inaccessible. The few books written for the layman are too simplistic to allow an understanding of the issues involved (inasmuch as they are technical rather than sociological or political), whereas the specialist monographs are incomprehensible, assuming a background which the layman does not have. Even for professionals, much of the information required to counter opposition arguments is scattered through the literature, and there has been no single book giving the necessary background technical information across the whole nuclear front.

*A Guidebook to Nuclear Reactors* was written specifically to try and overcome these problems. It is intended for both the non-technically trained and the professional needing a wide ranging but concise guide to the whole nuclear power field.

The book is divided into four parts. The first, entitled "A General Introduction to Nuclear Reactors", starts by outlining the main features of a nuclear power plant and then goes on to consider the effect which such a system has on its surroundings, in

terms of both heat and radioactivity discharges. At the end of this section there is a chapter on accidents, discussing how they can arise and what the consequences may be, and concluding with an introduction to risk assessment along the lines of the American Rasmussen Study.

In the second part there are four chapters, each dealing with a specific reactor type, namely pressurised water reactors, boiling water reactors, heavy water reactors and gas-cooled thermal reactors. Here each chapter has the same format, namely a description of the layout and principal components of each reactor type, followed by a description of auxiliary systems. Next comes a discussion of the safety systems incorporated in the reactor, and there is then consideration of fuel utilisation and reactor operation.

The third section, also of four chapters, starts off by considering the question of uranium resources and then goes on to discuss the role of both fast breeder reactors and high conversion efficiency thermal systems. The various nuclear fuel cycles are then outlined in the context of fuel processing, which leads naturally to a chapter on "The Weapons Connection". Finally, in the fourth section, there are three chapters on advanced reactor systems ("Breeder, Near-Breeder and What Not"), the last chapter including a mention of fusion, fission-fusion and accelerator-based nuclear fuel breeding systems.

Let it be said immediately that this is an extremely good book. However, the concentration on reactor systems found in, or of interest to, the United States means that systems in, for example, the USSR, together with our own in Britain, get scant mention ("In addition, England has constructed gas-cooled reactors with carbon dioxide coolant"). There is some repetition, which may have a didactic purpose, and some Americanese ("The style of the book is more informational than argumentative"), though mercifully little.

Nevertheless, faced with the abundance of extremely useful tables and figures, it is churlish to emphasise these faults. For the professional involved in nuclear debates and for the student entering the field this is an invaluable book. However, its value for the layman is likely to be limited by the sheer quantity of information and density of technical terms which, even when explained, need to be absorbed in order to appreciate the text. Nevertheless, as a source of background information for the non-scientist it remains the best book which I have seen, and Anthony Nero deserves credit for trying to raise the level of the so-called 'nuclear debate'. □

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# Transport organ physiology

O.H. Petersen

*Membrane Transport in Biology*. Vol. IV A and B. Edited by G. Giebisch, D.C. Tosteson and H.H. Ussing. Pp.471; 939. (Springer: Berlin, Heidelberg and New York, 1979.) Two volumes DM296; \$162.80.

RECENT progress in the study of transport has been astonishing. This is clear when reviewing the impressive series on *Membrane Transport in Biology*, which has now been brought to an end with volume IV (consisting of two books) dealing with the transport organs. The editor of this last volume, Gerhard Giebisch (Yale), is to be congratulated on providing a number of good chapters written by scientists who are among the leaders in their fields of study. It was also a good idea to have asked Renkin (California) to provide an introductory chapter on transport across capillary membranes. However, while there is no denying the general high quality of the individual contributions, this volume is marred by an unfortunate lack of balance. Of the 18 chapters, no less than 10 deal exclusively with the kidney. In contrast, there are only two chapters on the intestine. Salivary glands, gastric secretion, exocrine pancreas, gall bladder and liver cells have one chapter each. This is clearly not a reasonable balance either in terms of current research interests or with respect to general importance. Furthermore, it means disturbingly uneven presentation with regard to depth of treatment and level of detail. Thus, while Boulpaep (Yale) devotes 47 pages exclusively to a discussion of kidney electrophysiology, Schultz (Houston) uses 31 pages in the only chapter on the small intestine and Schulz and Ullrich (Frankfurt) have 41 pages at their disposal to deal with all aspects of pancreatic membrane transport biology, including the electrophysiology! Nevertheless, Schulz and Ullrich's chapter on the pancreas is a model of clarity and also seems more up to date than many others, betraying the fact that the greater part of these two books must have been finished in late 1976 or early 1977, although some more recent references seem to have been brought in at the proof stage.

The many chapters on kidney transport biology have their problems. In spite of separate chapters on electrochemistry (Khuri, Beirut) and electrophysiology, there is extensive repetition, particularly of the electrophysiology (even of some of the figures!) in chapters on sodium chloride (Windhager, Cornell) and potassium transport (Giebisch, Yale). One figure illustrating Na-glucose co-transport in membrane vesicles (data of Kinne,

Frankfurt) is shown three times (pages 156, 426 and 555). The interaction between transport of sodium and organic solutes, originally discovered by Weymouth Reid at the turn of the century, rightly features prominently both in the kidney section, in an excellent review by Ullrich (Frankfurt), and in sections on the intestine and the pancreas.

Immediately following the massive kidney sections is a comprehensive chapter on the salivary glands by Young (Sydney). It is perhaps surprising that salivary glands should be much more extensively treated (more than 100 pages) than the stomach (Forte and Machen, California) or the pancreas, but at least we have here a very complete (up to 1977) account of the transport physiology of these glands. The completeness is, however, somewhat at the expense of clarity. It seems unlikely, for example, that the non-specialist will find the

20 pages dealing with salivary gland electrophysiology (not a single illustration here!) easy going.

One cannot help wondering what readership the editor of this beautifully produced volume had in mind. The 10 very detailed kidney chapters must be attractive to nephrologists who might also enjoy the comparatively short but generally clear accounts of the other transport organs (or some of them; where is, for example, the placenta?). The title is, however, misleading, as it seems to announce a comprehensive treatment of membrane transport in the transport organs. What a pity that this last volume has only become a *Membrane Transport Biology* for nephrologists! □

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## Autoradiographic techniques

Tim Appleton

*Techniques of Autoradiography*. Third edition. By A.W. Rogers. Pp.429. (Elsevier/North-Holland: Amsterdam, New York and Oxford, 1979.) \$59; Dfl.133.

It is always a difficult thing to write a book covering a wide range of techniques. Rogers has tackled his task with an infectious enthusiasm and I admire the courage and hard work which has gone into this work. Unlike the earlier editions, this edition is conveniently split into three main parts; the theoretical basis of autoradiography (129 pages); the planning and interpretation of autoradiographic experiments (151 pages); and description of autoradiographic techniques (126 pages). There is also a useful appendix of data which any autoradiographer will appreciate.

I wish I could be enthusiastic about this book. As with earlier editions the descriptions of techniques are mingled in with the text. It would be much more helpful to the beginner and expert alike if detailed descriptions of techniques were set out clearly in the form of a schedule, in heavy type-face, so that they can be easily found and followed. The chapter on photomicrography is not as helpful as it could be, because the quality of the illustrations is very poor — worse than in the first edition. I suspect that this may be the fault of the publisher, as some of the figures which are 'identical' with those in the first edition are clearly at a higher magnification in the third edition, yet the captions still show the lower

magnification. Figure 54 in the third edition is of the same field as figure 31 of the first edition; both figures show a magnification of  $\times 135$ , yet the third edition plates are magnified a further 1.4 times. Such errors are frequent and should have been noticed by the author at the proof stage. Considerable clarity has been lost in many of the illustrations and some of the line drawings are so faint in the review copy that they became irritating. Captions are frequently placed in awkward positions and orientations.

References throughout were fairly complete but did not give titles. Occasionally I found that the reference quoted was not always the authoritative one but taken from a symposium report or review. This resulted in some inaccuracy which might confuse the reader.

The first edition of this book appeared in 1967, was 335 pages long with 77 illustrations. The third, "completely revised", edition has grown to 429 pages with 98 illustrations. I thought that the first edition was too long and my immediate reaction to the latest edition is much the same. However, there is often some benefit in discussing techniques in such a lengthy manner; I found my interests in autoradiography rekindled as I read on. Such length is only really acceptable if the price can be kept at a reasonable level, and I fear that a cost of around £30 will prove too high for the younger scientist.

It is inevitable that a book can never be up to date, and this is certainly true of this one. I would have liked to have seen more discussion of the use of scintillators to increase autoradiographic sensitivity, and on the localisation of diffusible substances at the level of the electron microscope. But then the book would have been even longer and, I fear, more expensive still. □

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